ACTIVATION AND MODULATION OF CELL-MEDIATED CYTOTOXICITY AGAINST TUMOURS

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ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
ALK	Activated lymphocyte killer
C'	Complement
CD	Cluster of differentiation
Con A	Concanavalin A
CP	Cytolytic protease
cpm	Count per minute
^{51}Cr	Chromium-51
CTL	Cytotoxic T lymphocyte
CY	Cyclophosphamide
DDW	Double distilled water
DG	Diacylglycerol
DMSO	Dimethylsulfoxide
E:T	Effector-to-target cell ratio
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EL-4	Benzopyrene-induced T cell lymphoma of C57BL/6J mice
H7	1-(5-Isoquinolinyl sulfonyl)-2-methylpiperazine
HIFCS	Heat-inactivated foetal calf serum
^{3}H-TdR	Tritiated thymidine
hr(s)	Hour(s)
ICAM	Intercellular adhesion molecule
IL-2	Interleukin-2
i.p.	Intraperitoneally

IP_3	Inositol 1,4,5-trisphosphate
IP_4	Inositol 1,3,4,5-tetrakisphosphate
i.v.	Intravenously
kD	Kilodalton
LAK	Lymphokine-activated killer
LFA-1	Lymphocyte-function-associated protein 1
LGL	Large granular lymphocytes
MBL-2	Moloney virus induced T cell lymphoma
mCi	Millicurie
μCi	Microcurie
2-ME	2-Mercaptoethanol
αmMan	α-Methyl-D-mannoside
MHC	Major histocompatibility complex
min(s)	Minute(s)
MMC	Mitomycin C
MTC	Macrophage-mediated tumour cytolysis
NEO	Neomycin
NK	Natural killer
NKCF	Natural killer cytotoxic factor
P815	Methylcholantrene-induced mastocytoma of DBA/2 mice
PBL	Peripheral blood leukocytes
PBS	Phosphate-buffered saline
PHA	Phytohaemagglutinin
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PMA	Phorbol myristate acetate
Poly I:C	Polyinosinic-polycytidylic acid

PSF	Penicillin-streptomycin-fungizone solution
S.E.	Standard error
sCon A	Succinyl Concanvalin A
T cell	Thymus-derived Cell
TNF	Tumour necrosis factor
TPA	12-o-tetradecanoyl-phorbol-13-acetate
W7	N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide
WEHI-3	Myelomonocytic leukemia of BALB/c mice
YAC-1	Moloney virus induced T cell Lymphoma of A/Sn mice

AIM AND SCOPE OF THIS THESIS

It has been well documented that cell-mediated killing by immune cells is an important defence mechanism against the proliferation of tumour cells. Over the last few years considerable work have been done in uncovering the mechanisms by which cytotoxic cells kill their tumour targets. The aim of this thesis project is to study how normal lymphocytes can be activated to kill syngeneic and allogeneic tumour cells of known antigenicity. Attempts will also be made to modulate their lytic potential in view of enhancing their ability to restrict the growth and dissemination of malignant cells.

In the work presented in this thesis, three different experimental approaches have been adopted for studying the activation and modulation of lymphocyte-mediated cytotoxicity against tumours. These include (1) the direct induction of lymphocyte-mediated killing against tumours by mitogenic plant lectins; (2) the nonspecific triggering of cytotoxicity against tumours by pharmacologic agents that can mimic antigenic stimuli in inducing transmembrane signalling in lymphocytes; and (3) the generation of 'tumour-specific' cytotoxic T lymphocytes by a combination of *in vivo* and *in vitro* immunization techniques. The philosophy of the present study is to enlarge our basic knowledge and understanding of the properties and the functional roles played by various types of cytotoxic lymphocytes in the host defence against malignant tumours. It is hoped that the manipulation of the cytotoxic response *in vitro* may provide us with better insights into the signal transduction mechanisms in cell-mediated cytotoxicity.

CHAPTER ONE : GENERAL INTRODUCTION

CONTENTS

The host immune system is composed of two main interacting branches : cell-mediated and humoral immunity. Cell killing by immune cells represents an important natural defence barrier against proliferation of tumour cells, virus-infected cells and other foreign invaders (Young and Cohn,1986). Natural killer (NK) cells kill a wide spectrum of tumour cell lines and virus-infected cells nonspecifically whereas cytotoxic T lymphocytes (CTL) are major histocompatibility complex (MHC)-restricted and have antigen specificity. There are also non-MHC restricted cytotoxic cells expressing T-cell antigens which are present among lymphocytes activated by lectins, alloantigens, drugs and other lymphokines. In this introductory chapter, the roles of cell-mediated cytotoxicity in the defence of host against tumour growth and metastasis will be reviewed. Moreover, the *in vitro* activation of cytotoxic lymphocytes and the multiple mechanisms of lymphocyte-mediated killing will also be introduced. Because of the vast literature in these areas, the discussion that follows is neither comprehensive nor referenced in details. Only those topics that are most pertinent to the work done in this thesis will be highlighted.

1.1 Cell-mediated cytotoxicity in the defence of host against tumour growth and metastasis

It is well known that the immune system is composed of two major lymphocyte populations : The B cells which are responsible for antibody production (humoral immunity) and the T cells which

are responsible for specific cell-mediated immunity. The main function of the immune system is the destruction or elimination of foreign antigens. It may also generate immunological memory so that the host will respond much faster in the second confrontation with the same antigen. On the other hand, it may lead to the development of immunological tolerance and suppression. For anti-tumour responses, the immune system may also play dual roles that it may try to eliminate or destroy the tumour cells on one end whilst it may also facilitate the growth and metastasis of tumour mass on the other end (Beverley, 1978; Naor, 1979).

The anti-tumour mechanisms are depicted in Fig. 1.1. Those mediated by humoral immunity include antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated lysis of tumour cells. The major effector cells involved in ADCC are the eosinophils, neutrophils, macrophages and killer (K) cells. On the other hand, thymus-derived (T) lymphocytes are the major cell type related to the cell-mediated immunity and can be subdivided functionally into four classes : helper Tcells, suppressor T cells , cytotoxic T cells and delayed type hypersensitivity T cells. Additionally, nonspecific lymphoid cells including K cells, or null (non-T, non-B) cells, macrophages , lymphokine-activated killer (LAK) cells and NK cells also play a role in anti-tumour responses. However, in the following section, my discussion will mainly be focused on the properties of a few major cell types participating in the cytolysis of tumours.

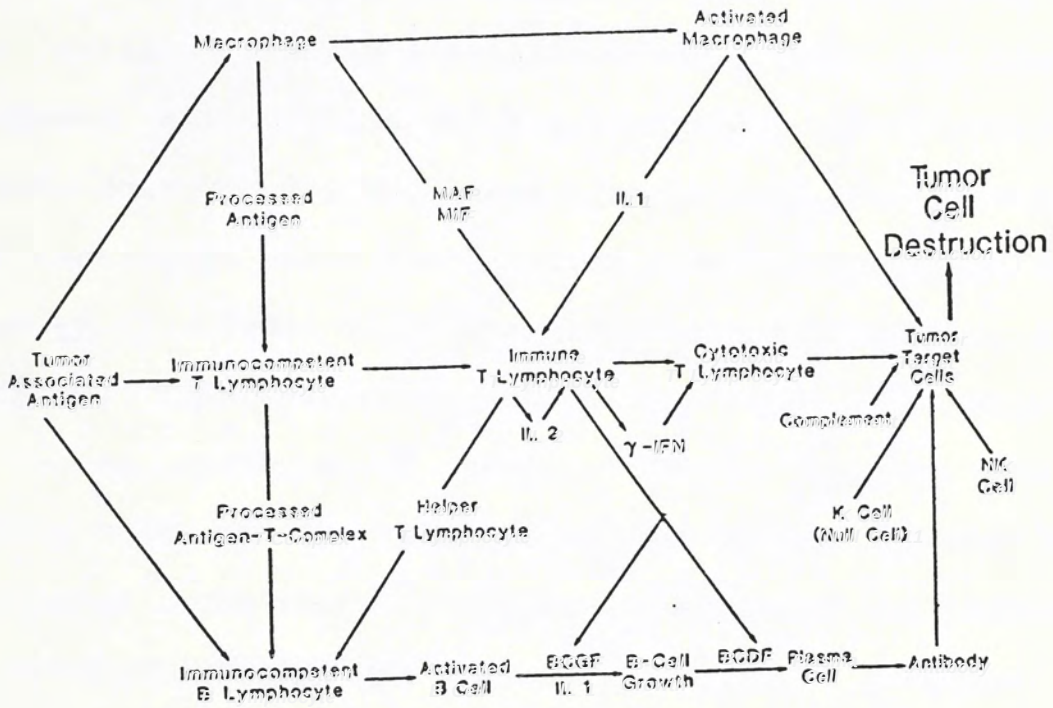

Fig. 1.1 Immunologic mechanisms involved in tumour cell destruction (Siegel, 1985).

1.1.1 Cytotoxic T lymphocytes :

Cytotoxic T lymphocytes have long been implicated as the prinicipal effector cells in allograft rejection (Berke, 1980), tumour immunity (Cerottini and Brunner, 1974) and lysis of virus-infected cells (Ting and Bonnard, 1976). CTL are the best characterized killer cells which are antigen- specific and MHC-restricted. Most CTL are MHC class 1 restricted, but some are MHC class 2 restricted and the function of these cells is unclear (Braakman et al., 1987; Shinohara, 1987). Moreover, some cloned, interleukin-2 (IL-2)-dependent $CD3^+$ T-cell lines are reported to lyse a broad spectrum of tumour targets without the MHC restriction although they also recognize targets via the CD3/Ti complex since both anti-clonotypic and anti-CD3 antibodies can inhibit cytolytic activity. They also rearrange Ti genes. Some also recognize an activation antigen, designated as TNKar or 4F2 on target cells. This type of cytotoxic T lymphocytes has been proposed by Lanier and Philips (1986) as a unique type of CTL - non-MHC restricted cytotoxic T lymphocytes. This type of CTL may also include effector cells that are involved in lymphocyte-mediated cytotoxicity induced by lectins or phorbol esters and/or calcium ionophores as well as the lymphokine-activated killer activity.

The CTL-mediated lysis of a specific target can be divided into three phases (Tschopp and Jongeneel, 1988) :
1. Recognition and binding : CTL are first adhered to their target cells nonspecifically by two CTL-cell adhesion molecules on the cell surface : LFA-1 (lymphocyte -function -associated

protein 1), a heterodimer consisting of an alpha subunit of 180 kD and a noncovantly linked beta subunit of 95 kD , and CD2 (cluster of differentiation), a 45-50 kD glycoprotein. These molecules interact with the two target cell surface proteins called ICAM (intercellular adhesion molecule) and LFA-3 respectively. ICAM is poorly defined at the molecular level. LFA-3 has a molecular weight of 55,000-70,000. This interaction is Mg^{++}-dependent and strengthened by interactions between the T cell receptor complex (Ti/CD3) on the CTL and a combination of its cognate antigen and major histocompatibility complex coded molecules on the target cell. The majority of CTL is class-1 MHC restricted. The T cell receptor consists of the Ti alpha/beta heterodimer which carries the clonal specificity through its variable regions and the associated CD3 complex. CD3 appears to be involved in the post-recognition activation step (Spits et al., 1986). The avidity of these cell-cell interactions is further enhanced by the binding of CD8 molecule (mouse Lyt-2) to monomorphic determinants of the appropriate MHC molecule on the target cells.

2. 'Lethal hit' delivery : Occupation of the CTL causes a local Ca^{++}influx (Weiss et al., 1986; Phoenie et al., 1987) which acts as a signal for the 'lethal hit' delivery stage. It leads to a drastic morphological reorganization of the CTL and the subsequent delivery of molecules 'lethal' to the target cell. Once the lethal molecules have been released from the CTL, the CTL then recycle and go on to kill other targets. The lytic molecules will be discussed later in this chapter.

3. Target cell lysis : CTL-mediated lysis of target cells is characterized by the dual disintegration of DNA and plasma membrane of target cells. The disintegration of DNA is not observed in complement-mediated lysis. During the lytic stage, massive Ca^{++} influx and changes in the overall shape of the target cells are followed by dilation of the endoplasmic reticulum and chromatin condensation (Duvall and Wyllie, 1986). These morphologic changes are accompanied by DNA fragmentation (Duke et al., 1983) and finally, the target cells release macromolecules into the extracellular environment due to cytoplasmic membrane damage.

1.1.2 Natural killer cells :

Natural killer cells represent a heterogeneous population of lymphoid cells that generate spontaneous cytotoxic activity against tumour cells as well as virus-infected cells in vitro (Kiessling and Wigzell, 1979). NK cells are discovered in the mid-1970s and soon recognized as potentially important in the protection against tumour cells and infections, and in immunoregulation (Moller, 1979 ; Welsh, 1981; Herberman, 1982). Moreover, NK cells may also be involved in allogeneic lymphocyte-mediated cytotoxicity (Fossum and Rolstad, 1986).

NK cells are different from other immune cells by their morphology, surface phenotype, antigenic markers and target cell specificity (Ortaldo, 1986). In contrast to cytotoxic T cells, NK cells have no MHC restriction. They can kill a variety of

syngeneic, allogeneic and xenogeneic tumour cells as well as some fetal cells, virus-infected cells, chemically-treated cells and subpopulations of normal lymphoid or hematopoietic stem cells. The cells that are most susceptible to NK lysis *in vitro* seem to be those lacking MHC antigen (Karre *et al.*, 1986). In human system, NK activity is mediated by the large granular lymphocytes (LGL) that are present in about 5 - 10 % of the peripheral blood mononuclear cells (Saksela *et al.*, 1979; Timonen *et al.*, 1981). NK cells also express a few common surface markers as macrophages and also several T cell-associated markers. In the human system, NK cells lack CD3 surface marker but express CD11 and T-related markers (CD2, CD8 , OKT10) (Lanier *et al.*, 1985; 1986). In the murine system, NK cells lack Lyt2 and L3T4 T cell markers but express the asialo GM1, Ly5, Qa5, NK1 and NK2 on the cell surface (Jondal, 1987). In addition, a subset of mouse NK cells expresses Thy 1 (Wiltrout *et al.*, 1984). Moreover, NK cells are nonadherent, nonphagocytic cells and express surface receptors for the Fc portion of IgG (CD16) and thus can mediate an ADCC reaction. NK cells originate in the bone marrow and exhibit a characteristic organ distribution. The activity declines from peripheral blood, spleen, lymph nodes and bone marrow and does not present in thymus (Herberman *et al.*, 1975; Kiessling *et al.*, 1975). Moreover, NK activity is age-dependent and in the mouse it is low during the first 3 weeks, peaks at 6-10 weeks old, and gradually declines thereafter.

Unlike CTL, NK activity does not appear to be dependent on antigenic sensitization and no secondary memory response has been

seen (Herberman, 1974). However, NK activity can be activated in vivo by a variety of bacterial agents as well as synthetic agents such as polyinosinic polycytidylic acid (poly I:C) and pyran copolymer . In addition, they also respond to T-cell mitogens, IL-2 and interferons *in vitro*.

Although different aspects of NK cells such as morphology, phenotype, mode of function and specificity have been extensively studied, the important question of target cell recognition has not been resolved (Lanier and Philips, 1986). The expression of CD2,CD16 and adherence type receptors such as CD11a/CD18 on the NK cell surface may be somehow related to the recognition process. It has been suggested that NK cells express CD2 receptors in such steric configurations that they both bind and activate cells in combination with supporting receptors such as CD16, CD11a/CD18 and possibly others. The postulated dual binding and triggering function of CD2 receptors on NK cells is based on the demonstration of different functional epitopes within the CD2 receptor molecule (Bernard *et al.*, 1986; Meuer *et al.*, 1986). On the other hand, there is a double restriction in NK cell specificity involving two independent but sequential stages in recognition represented in binding and triggering by high mannose containing and asparagine-linked oligosaccharide on the tumour target cell surface (Kiyohara, 1987.)

After recognition of target cells, the next step involves the triggering of intracellular processes : rearrangement of the

granules and other cytoplasmic organelles towards the site of binding with the target cells. Finally, NK cells lyse the target cells by secretion of cytolysin (Schmidt et al.,1986) as well as other lytic molecules. Moreover, natural killer cytotoxic factor (NKCF) released by NK cells during the interaction of spleen cells with NK-susceptible targets may also be involved in the mechanism of lysis by NK cells. NKCF appears to be relatively unstable proteins with the molecular weight in the range of 20,000-40,000 (Farram and Targan , 1983). Moreover, NKCF exhibits some target cell specificity. Besides, it can lyse targets in the absence of Ca^{++} and requires 8-12 hours of incubation at 37 °C but not at 22 °C (Deem et al., 1986).

1.1.3 Macrophages :

Macrophages as well as polymorphonuclear leukocytes constitute the first line of defense of the body against most infections. Macrophages contribute at several stages in the specific immunity leading to antibody production and cell-mediated immunity directed against specific antigens. Moreover, macrophages also exert a nonspecific anti-bacterial action with the co-operation of nonspecific opsonins and recognition factors. Many important biological macromolecules such as components of complements, transferrin, interferon, arginase and hydrolytic enzymes such as lysozyme and collagenase are secreted massively by macrophages. Moreover, macrophages also scavenge tissue debris, detoxicify certain chemical substances and also participate in the metabolism and disposition of iron, lipids and

proteins (Neveu, 1986).

In anti-tumour mechanisms, the macrophages play important roles in both the afferent and efferent arms of the immune system. Macrophages can act as antigen-presenting cells which modify and present tumour-specific antigens to T-lymphocytes with MHC restriction. Moreover, macrophages can lyse the tumours directly (macrophage-mediated tumour cytolysis, MTC) or through ADCC. In the MTC process, the cytolysis involves two main steps. First, the macrophages bind to the tumour targets through nonspecific receptors. Then, the macrophages are activated and secrete large amount of various cytolytic mediators such as cytolytic protease (CP), arginase, tumour necrosis factor (TNF), interferon and hydrogen peroxide. Among them, CP is considered to be the major lytic molecule in the MTC system (Espevik et al., 1986). In ADCC system, hydrogen peroxide is reported to be the major cytolytic agent. However, all mediators may be involved in the lytic process and the co-operation of them may induce a synergistic effect. Nonspecifically, macrophages can be activated by different agents such as endotoxin, double-stranded RNA to inhibit the growth of lymphoma and sarcoma cells in vitro or even lyse some malignant tumour targets. Although many reports showed that macrophages indeed play a role in anti-tumour mechanism, macrophages are also shown to mediate the immunosuppression of host lymphocytes which may limit the anti-tumour activities and enhance tumour growth.

1.1.4 Cell-mediated cytotoxicity via 'conferred specificity': Antibody-dependent cellular cytotoxicity (ADCC)

Although cytolytic action can be triggered by the specific interaction between effector cells and target cells (i.e. via CD3/Ti complex or the unidentified NK target receptor), each cell type may also possess alternative pathways through which target specificity may be 'conferred' exogenously. In ADCC, antigen specificity can be conferred by specific IgG antibodies directed against antigens on the surface of target cells. The effector cells should thus express Fc receptors for the IgG molecules. These include a variety of cell types : K cells, macrophages, neutrophils, eosinophils, NK cells and $CD3/Ti^{+}$ CTL. The mechanism of killing by ADCC effectors may differ from that of the CTL and NK cells and will not be discussed here (for review, see Siegel, 1985)

1.2 Activation and modulation of lymphocyte-mediated cytotoxicity against tumours

It is well known that some weakly immunogenic tumour cells may not induce a high enough level of immune response *in vivo*. Therefore, in order to enhance the anti-tumour cell-mediated immune responses, different methods have been utilized to induce a higher level of cell-mediated cytotoxicity *in vitro*.

1.2.1 Induction of nonspecific lymphocytes-mediated cytotoxicity by lectins

Non-MHC restricted cytotoxicity against tumours can also be mediated by CTL in the presence of certain lectins that are mitogenic for T cells such as concanavalin A (Con A) and phytohemagglutinin (PHA). It has been demonstrated that cloned, antigen-specific, MHC-restricted CTL can lyse inappropriate tumour targets in the presence of lectins (Bonavida et al., 1983). Similarly, alloimmune cells or lectin-activated splenocytes can kill nonspecifically a wide variety of syngeneic or autologous cells in the presence of mitogenic lectins such as Con A and PHA (Forman and Moller, 1973; Bevan and Cohn, 1975; Bradley and Bonavida, 1978). This has been termed as lectin dependent cellular cytotoxicity (LDCC). On the other hand, these lectins do not induce or augment the cytotoxic activity of CD3+ NK cells (Lanier and Phillips, 1986). It has been suggested that lectins mimic specific antigen by interaction with the CD3/Ti complex and cause the initiation of the cytolytic mechanism (Hubbard et al., 1986). However, only activation of effector cells by lectins is not enough to induce lysis of target cells as it has been demonstrated that activated effector cells do not lyse target cells when the binding of CTL and target cells is inhibited (Green, 1982). Moreover, bridging alone is insufficient for lysis to occur because non-mitogenic lectins such as soybean agglutinin (SBA) and wheat germ agglutinin (WGA) do not mediate LDCC though they can cross-link CTL and target cells (Parker and Martz, 1980). Thus, in LDCC the lectin may have a dual role : 1. formation of a lectin bridge between

effector cells and target cells and 2. activation of effector cells. However, it has been found that optimal lysis was obtained when Con A-treated target cells were allowed to react with untreated CTL, but not when Con A-treated CTL were reacted with untreated target cells (Berke et al., 1981a,b). Similar results were obtained with PHA. Besides, recent results showed that MHC-deficient target cells were resistant to LDCC and it was not due to the inability of the target cells to bind lectins (Keren and Berke, 1983). Moreover, it has been found that lysis in LDCC can be blocked efficiently by antisera directed against target cell MHC-antigens (Ag), but only poorly by sera against non-MHC-Ag (Berke et al., 1981b). Therefore, these results suggest that the lectins may bind to target cell MHC antigens, and perhaps other cell surface determinants to render the target cell nonspecifically recognized by CTL receptors. The ability of monoclonal antibody which is directed against the T-cell antigen receptor to inhibit both antigen and Con A-mediating lysis (Sarmiento et al., 1982; Hubbard et al.,1986) further supports this hypothesis. Moreover, it has been shown that papain-sensitive, N-linked glycosylated molecules on the target cell surface must be present for LDCC to occur but not for direct MHC-specific CTL killing to take place (Gorman et al., 1987). It has been suggested that papain-sensitive molecules involved are the class 1 MHC proteins. Therefore, lectins such as Con A may act on a glycosylated papain-sensitive component(s) to render the target cell susceptible to CTL-mediated killing.

LDCC is known to be both calcium and temperature -dependent. Optimal lysis only occurs at a certain concentration of lectin . Higher concentrations of lectins (e.g., 100-250 µg/ml Con A) showed inhibition of nonspecific killing (Tartof, 1980). LDCC can also be inhibited by metabolic inhibitors and α-methyl-D-mannoside (Berke, 1983).

1.2.2 Induction of nonspecific lymphocyte-mediated cytotoxicity by phorbol esters and/or calcium ionophores

Cloned murine cytolytic T lymphocytes as well as normal human lymphocytes can be triggered by the phorbol ester and calcium ionophore to lyse a wide variety of tumour target cells efficiently (Ju et al., 1987; Lancki et al., 1987; Mikael et al., 1987). Neither phorbol ester nor calcium ionophore alone induce efficient lysis. Phorbol ester alone has shown to have the capability of inhibiting the lytic activity of cloned T cells (Russell, 1984) and NK cells (Abrams et al., 1983) as well as lectin- and antibody-dependent killing (Jondal et al., 1986). However, phorbol ester can also induce cloned and in vivo-produced CTL to lyse weak and nonspecific target cells (Russell, 1986). It may seem paradoxical that a drug which is an activator of protein kinase C can have an opposite effect on a system that may depend on the expression of the same enzyme. However, it should be noted that phorbol ester can rapidly down-regulate cell surface receptors such as CD4 and Fc receptors for IgG (Solbach, 1982). Moreover, it can also induce a negative feedback inhibition of the hydrolysis of phosphatidylinositol 4,5-

bisphosphate(PIP_2) and thus prevent further formation of inositol phosphates and diacylglycerol (DG) (Baraban et al., 1985). Such feedback inhibition is also linked to a block of Ca^{++} uptake (Eisenberg et al., 1985). Thus, the effect of phorbol ester on the lytic ability of cells seems to be determined by the dose used and the duration of exposure of cells to the drug. For calcium ionophore, it is also unexpected that it is suppressive for NK killing even if such killing is considered to depend on an active Ca^{++} uptake. Phorbol ester can mimic DG actions to stimulate protein kinase C and calcium ionophore can increase intracellular Ca^{++} levels. It has been demonstrated that the early steps of murine lymphocyte activation can be bypassed by a synergy between calcium ionophore and phorbol ester (Truneh et al., 1985). Moreover, only the combination of the two drugs can induce IL-2 secretion and expression of IL-2 receptors by T lymphocytes (Truneh et al., 1985). With cytotoxic T cell clones, calcium ionophore and phorbol ester can substitute for antigen in the induction of killer cells from primed precursors (Alber et al., 1985; Isakov and Altman, 1985). The characteristics of the lytic process induced by the two drugs are similar to that of antigen-specific or lectin-induced cytotoxicity by CTL. Lysis is both temperature and calcium-dependent. Besides, phorbol ester and calcium ionophore can induce both NK cell-enriched, T3-negative cells and NK cell-depleted, T3-positive cells to lyse tumour targets (Mikael et al., 1987). Moreover, the drug-induced killing did not increase the optimal lectin and antibody-dependent killing (Mikael et al.,1987).

1.2.3 Effects of lectins and drugs on the signal transduction in T lymphocytes

T lymphocyte activation and proliferation can be initiated by interaction between an antigen associated with MHC molecule or other ligands such as mitogenic plant lectins or drugs and their corresponding cell surface receptors (Fig. 1.2). Recent results showed that inositol phospholipid hydrolysis is important in T-cell activation. In this pathway, interaction of a ligand with its cell surface receptor stimulates a phosphoinositide-specific phosphodiesterase (phopholipase C) to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid normally found in the cell membrane in minute quantities. GTP-binding proteins may constitute the coupling mechanism between receptor occupancy and phospholipase C stimulation. The hydrolysis products are diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). These two second messengers act synergistically to elicit a physiological response (Isakov et al., 1986). DG activates protein kinase C (PKC) by increasing its affinity for Ca^{++}. Protein kinase C is a single 76-80 kD polypeptide consisting of two domains : a hydrophilic domain which possesses the catalytically active center, and a regulatory hydrophobic domain which binds to Ca^{++}, DG and phospholipid. Upon activation, PKC is translocated from the cytosol to the membrane and catalyses the phosphorylation of numerous protein substrates at seryl and threonyl residues by using ATP as a phosphate donor. Moreover, PKC also stimulates Na^+/H^+ exchanger and thus leads to an increase in intracellular pH which may be a critical event in cell activation and growth. The another

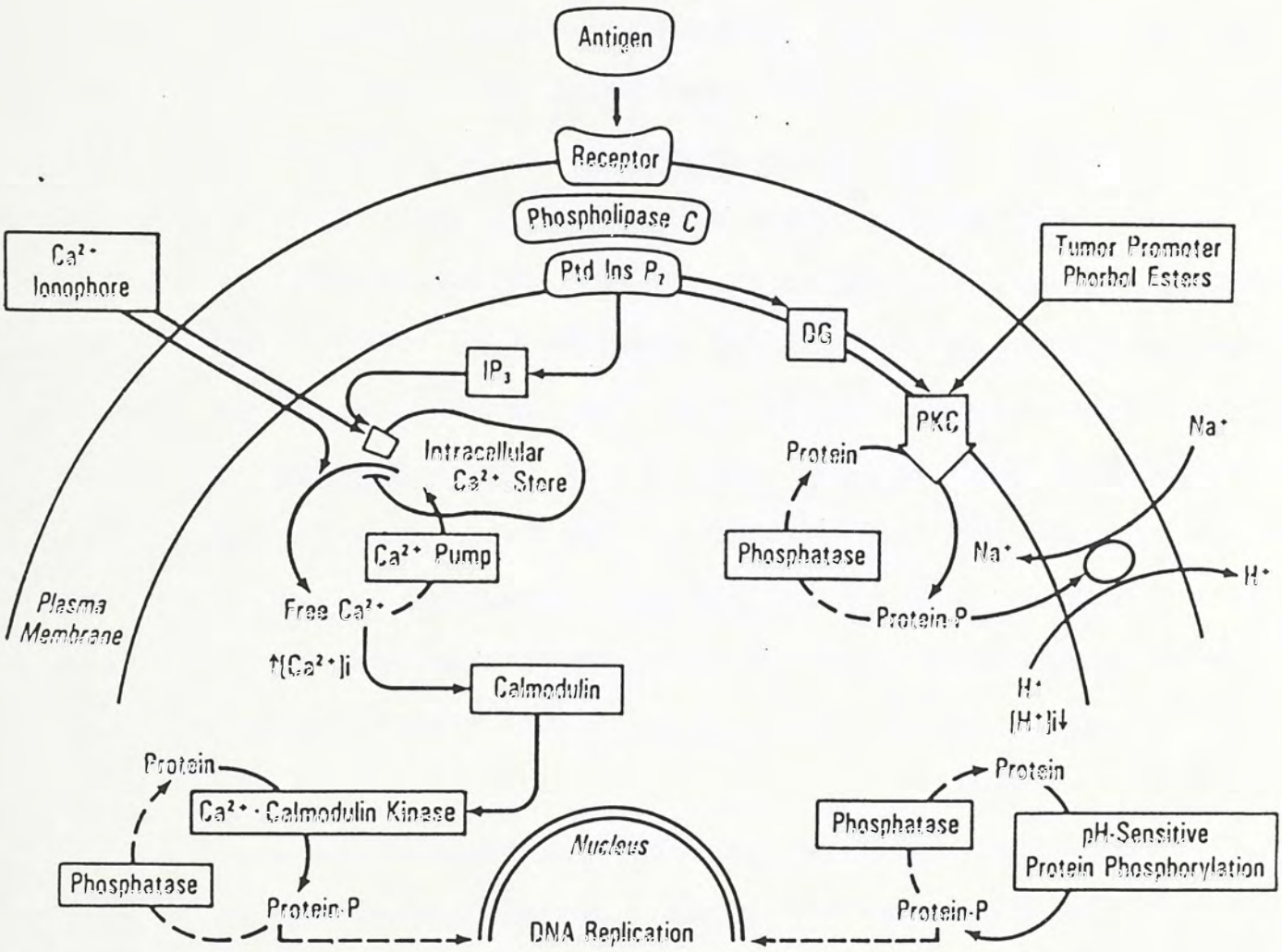

Fig. 1.2 A model of transmembrane signal transduction and the early intracellular events in T lymphocytes after activation (Isakov et al., 1986).

second messenger IP_3 releases Ca^{++} from the endoplasmic reticulum and thus increases the concentration of free intracellular Ca^{++} . IP_3 may bind to a specific receptor (Spat et al., 1986) and opens a calcium channel (Muallem et al., 1985) on the endoplasmic reticulum through a guanine regulatory mechanism (Gill et al., 1986). IP_3 can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate (IP_4) (Irvine et al., 1986) which can act synergistically with a fall in the intracellular calcium pool to stimulate calcium influx from the extracellular space (Taylor, 1987). This event may activate Ca^{++}-dependent protein kinases consisting of calmodulin, Ca^{++},and protein kinases resulting in protein phosphorylation. As a result, cell surface receptors for IL-2 appear and the T cell progresses into the mitotic cycle because of IL-2 production and binding to IL-2 receptor. This signalling pathway in T lymphocytes has been described in details (Gelfand et al., 1987; Isakov et al., 1987; Linch et al., 1987).

Lectins are carbohydrate-binding multimeric proteins derived from plant and animal source. Among the various plant lectins, Con A is best characterized as a mitogenic protein. Con A activates T-lymphocytes by binding to a cell surface receptor CD3 antigen without entering the cells (Ballas et al., 1981). Only multimeric Con A can stimulate T cells. Monomeric fragments of Con A are unable to stimulate T cells though they can bind to them. Since Con A is multimeric,, it can crosslink receptors between cells leading to cell agglutination (Gunther et al., 1973). The cellular reactions mediated by Con A can be divided

into the immediate events which occur in seconds to minutes and the delayed events which occur in hours or days (Ashman, 1985). The immediate events include : Con A receptor capping and patching; rapid changes in K^+ and Na^+ fluxes; activation of membrane methyltransferases and phospholipase A2 ; phospholipid synthesis and turnover; arachidonic acid metabolism; calcium influx; cyclic nucleotide changes including cAMP and cGMP; protein phosphorylation; activation of serine esterases and increases in membrane transport rates for small molecules. The delayed events refer to the mitogenic actions of Con A on lymphocytes. These include acceleration of protein synthesis, RNA synthesis, polyamine synthesis; changes in carbohydrate metabolism; blast transformation and finally DNA synthesis (Robert, 1985). Recently, it has been shown that DNA synthesis depends critically on the presence of Con A during the first 3 hrs (phase I) and also during a second period around 16 - 24 hrs (phase II) whose exact timing may vary between lymphocyte subpopulations. In the interim period between 3 - 16 hrs, the presence of Con A has little impact on DNA synthesis. The calcium ionophore A23187 can substitute for Con A during the first period but not the second indicating that the calcium influx induced by Con A in phase I is a critical activation event.

Phorbol esters such as phorbol-12-myristate-13-acetate (PMA) and 12-o-tetradecanoyl-phorbol-13-acetate (TPA) can mimic the effects of DG and activate PKC without inducing an increase in intracellular Ca^{++} level. On the other hand, calcium ionophores can mimic the physiological signal of IP_3 and increase the

intracellular Ca^{++}levels. The two drugs can act synergistically on T-cell activation.

Phorbol esters are similar to DG in structure and can replace DG directly to activate PKC in vitro. The primary site of their action is specific receptors on the cell membrane and they initiate biochemical reactions that result in cell activation, proliferation, differentiation and transformation (Yuspa et al.,1976; Touraine et al., 1977; Lotem and Sachs, 1978; Blumberg, 1981). PKC is considered to be the cellular receptor for them. Phorbol esters can stimulate proliferation of resting human T cells (Isakov et al., 1986 ; Isakov and Altman, 1987). They induce expression of IL-2 and transferrin receptors. The proliferation of T cells is related to PKC activation. Moreover, this proliferation is not accompanied by IL-2 gene transcription or IL-2 secretion. Therefore, it may indicate that PKC is centrally involved in the transduction of activation signals in T cells and in the regulation of IL-2 receptor induction and expression. Moreover, the activation of PKC by phorbol esters leads to phosphorylation of the IL-2 receptors and the T3 complex in human T cells. On the other hand, phorbol esters can also downregulate T3 expression on human T cells (Cantrell et al., 1985; Isakov et al., 1985b; 1986a). It is likely that PKC also mediates negative feedback regulation of T3 expression. Besides, it has been reported that TPA can act synergistically with plant lectins such as Con A and PHA on inducing the production and secretion of IL-2 and other lymphokines by T cells.

Increased intracellular Ca^{++} level is a critical event in both signal transduction by various cell surface receptors and T-cell activation. Antigens, anti-CD3 and anti-T11 antibodies as well as calcium ionophores can stimulate intracellular Ca^{++} level increase and thus T-lymphocyte proliferation under appropriate conditions. The physiological intracellular (Ca^{++}) in resting cells is normally too low for PKC activation. However, binding of DG or phorbol esters can increase the affinity of PKC for Ca^{++} and thus allowing it to be partially or fully activated by basal intracellular Ca^{++} level. Alternatively, in the absence of DG or phorbol ester activation, PKC could be activated by increasing basal intracellular Ca^{++} level by calcium ionophore. At optimal concentrations, calcium ionophore alone can trigger IL-2 receptor expression and human T-cell activation without IL-2 production. Although phorbol esters and calcium ionophores can activate T cells independently, they can act synergistically on T-cell activation at non-mitogenic concentrations and induce high levels of IL-2 production (Chopra et al., 1987). It may be concluded that PKC activation is a sufficient signal for IL-2 receptor expression whereas activation of the IL-2 gene requires both activated PKC and a Ca^{++} signal (Truneh et al., 1985). However, the signals for DNA synthesis generated by binding of IL-2 to IL-2 receptors are different from those for IL-2 production and IL-2 receptor expression induced by phorbol esters and calcium ionophores (Koysau et al., 1987; Albert et al., 1988).

1.2.4 Lymphokine activated killer cells : nonspecific killers

Lymphokine activated killer cells can be generated by incubation of spleen cells or peripheral blood leukocytes (PBL) from normal or tumour-bearing individuals with IL-2 *in vitro* for 3-5 days. They can kill a wide variety of NK-resistant and fresh autologous tumour targets (Grim *et al.*, 1982) with no activity against normal cells (Grim and Rosenberg, 1984). LAK cells are indistinguishable from T lymphocytes in terms of surface markers since both of them express OKT-3, OKT-8, Leu-1 and 4F2 (Grim *et al.*, 1983) and have no universal markers in comparison to NK cells. However, the exact relationship between LAK and NK cells is controversial. At least two different LAK precursors are found and can be defined by their buoyant density, by their surface markers and by their susceptible target cells : one that is $Thy\text{-}1^-$, $Lyt\text{-}2^-$, $AGM1^+$ (i.e., NK-like cell) and one that is $Thy\text{-}1^+$, $Lyt\text{-}2^+$, $AGM1^+$ (i.e., T-cell-like) (Ballas *et al.*, 1987). The biochemical mechanisms that regulate LAK cell activation and the molecular events that accompany IL-2 stimulation of this process is not clear. However, the inhibition of adenylate cyclase in human cells through receptors coupled to the enzyme through G1 or indirectly via protein kinase C by pharmacologic agents could potentiate the effect of IL-2 on differentiation of LAK cells as well as lymphocyte proliferation (Beckner and Farrar, 1988). It may indicate that cAMP may play a role in regulating LAK activation.

Adoptive immunotherapy using LAK cells and recombinant IL-2 has been shown to be successful in the treatment of established

pulmonary or hepatic metastases as well as intraperitoneal tumours in murine models (Eggermont and Sugarbaker, 1987; Ottow et al., 1987). Preliminary results of human clinical trials have also been encouraging (Kimoto and Taguchi, 1987). However, repeated cycles of IL-2 plus LAK cells showed no additive anti-tumour effect. It has been suggested that antibodies directed against absorbed antigens on LAK cells and serum inhibitor of IL-2 are generated following repeated challenge (Eggermont and Sugarbaker, 1987).

1.2.5 Specific cytotoxic T cells generated by *in vitro* immunization

For adoptive cancer immunotherapy, cytotoxic cells can be produced massively by co-culturing effector cells with specific stimulator cells *in vitro* (Shu *et al.*, 1986). Although both allogeneic and syngeneic *in vitro* immunized lymphocytes are effective in preventing tumour growth *in vivo*, the use of allogeneic cells for adoptive transfer encountered the problems that they will be eliminated by the host immune defense system due to different MHC complex. Therefore, syngeneic lymphoid cells from normal (Kedar *et al.*, 1978 ; Mokyr *et al.*, 1978), tumour-immune (Cheever *et al.*, 1977,1978; Fernandez-Cruz *et al.*, 1979), or tumour-bearing (Mokr *et al.*, 1978; 1979) animals have been studied for their ability to generate tumour-specfic cytotoxicity by *in vitro* immunization techniques. These studies demonstrated that although *in vitro* immunized spleen cells from

normal animals were able to suppress tumour growth in vivo in the local adoptive transfer assay, they had only little effect when administered systemically to animals just prior to or shortly after the injection of tumour cells (Bernstein, 1977; Burton and Warner, 1977). On the other hand, in vitro immunized spleen cells from tumour-immune animals were able to suppress tumour growth both in the local adoptive transfer assay and when administered systemically to animals prior to the injection of tumour cells or 1 day following the injection of tumour cells.

In order to enhance the cytotoxicity of specific killer cells generated by in vitro immunization techniques, both the culture conditions and the composition of effector cells can be modified. The success of an in vitro immunization culture depends on : 1. the presence of 1×10^{-4} to 1×10^{-5} M 2-ME; 2. the presence of an appropriate percentage of macrophages; 3. the use of an appropriate batch of foetal calf serum; 4. a suitable ratio of responder-to-stimulator cells; 5. an appropriate duration of culture; 6. an appropriate density of lymphoid cells and stimulator cells ; and 7. the selection of an appropriate dose of irradiation or drugs used to inactivate the stimulator cells.

Moreover, the anti-tumour cytotoxicity upon in vitro immunization can also be augmented by modifying the composition of effector cells. These include : 1. increasing the frequency of cytotoxic T lymphocyte precursors (Wagner et al., 1972; 1973); 2. removal of suppressor cells. The methods used for removal of the suppressor elements depend on their nature.

Suppression by T cells may be eliminated either by the use of anti-I-J serum *in vitro* or by treatment of spleen cell donors with cyclophosphamide (CY) *in vivo*. On the other hand, suppression by macrophages can be eliminated either by glass adherence or by inactivation of macrophages with indomethacin , silica or carrageenan. Other methods include : 1. administering immunostimulants such as BCG to spleen cell donors; 2. adding immunostimulants such as BCG, Con A or interleukin 2 to the culture media and finally 3. increasing the immunogenicity of stimulator tumour cells by chemical or enzymatic modifications.

There are many advantages of utilizing *in vitro* immunized lymphoid cells compared to *in vivo* immunized cells. These include : 1. much higher anti-tumour cytotoxicity of effector cells can be generated; 2. weakly immunogenic tumours can be used to induce higher level of cell-mediated cytotoxicity; 3. the risks of injecting malignant cells into the host can be avoided and 4. a more rapid generation of cytotoxicity occurs.

Moreover, the effectiveness of adoptively transferred *in vitro* immunized lymphocytes for the therapy of tumour bearers has previously been restricted by the limited numbers of cytotoxic cells that are available. With the establishment of cultures of cytotoxic cells that continuously proliferate *in vitro* in the presence of IL-2, the cytotoxic cells could then be administered repeatedly in large numbers and thereby might increase the potential effectiveness of adoptive immunotherapy. In addition, specific cytotoxic T cells which only lyse those tumour cells

carrying a specific antigen can be selected and propagated due to the development of T-cell cloning techniques. Therefore, highly specific cytotoxic T cells which can only recognize a certain antigen on the tumour cell surface can be generated and used for adoptive immunotherapy.

1.3 Mechanisms of cell-mediated cytotoxicity

Cell-mediated cytotoxicity is an important defence mechanism against proliferation of tumour cells and other foreign agents. However, the lytic mechanism of cytotoxic lymphocytes is not clear yet. Recent studies showed that insertion of functional pores into target cell membrane may be involved in CTL and NK cell-mediated killing (Young and Cohn, 1986). According to this view, killing can be divided into four discrete events : 1. receptor-mediated binding which involves recognition of the target cell and interaction of effector and target cell surface; 2. polarization of Golgi apparatus, cytoplasmic granules, and other cytoskeletal elements towards the target cell; 3. exocytosis of granules and release of a pore-forming protein (PFP) called perforin/cytolysin into the intercellular space; 4. Ca^{++} -dependent assembly of membrane lesions by polymerization of PFP in the membrane. The net result is cell death due to colloidal osmotic lysis.

This model requires for a directed and localized degranulation of PFP into the contact zone between the effector and target cells. This is supported by the following observations : 1. unidirectionality of the killing process was seen such that

a CTL could lyse only one target cell at a time even though several target cells were bound to it (Zargury et al., 1979); 2. extensive apposition of plasma membranes occurred in the form of a network of membrane interdigitations at the sites of cell contact (Rosenau et al., 1981); 3. a rapid cytoplasmic reorientation of the granules, microtubule organizing center and Golgi apparatus occurred following effector-target conjugate formation (Kupfer and Dennert, 1984) but not observed when CTL were bound to nonlysable targets. In addition, fusion of granules directly with plasma membranes can be monitored continuously by high-resolution cinemicroscopic studies (Yannelli et al., 1986). Similar observations have been observed for NK-mediated killing and ADCC.

Moreover, many CTL and NK cells are found to contain electron-dense granules in their cytoplasm, commonly observed on the trans side of the Golgi (Henkart, 1985; Podack, 1985). Granules isolated from these cells lyse tumour targets nonspecifically and produce circular lesions with an internal diameter of 160 Å on the target cell membrane. The lytic protein responsible for these lesions has recently been purified and termed as pore-forming protein or perforin/cytolysin. It migrates on SDS-gels with a molecular mass of 70-75 kD (under reducing conditions) or 60-66 kD (under nonreducing conditions) (Masson and Tschopp, 1985; Podack et al., 1985; Henkart et al., 1986). The ability of perforin to form functional pores in target membranes was definitely demonstrated through reconstitution

experiments (Young et al., 1987). PFP can depolarize the membrane of cultured cells and tumour cells nonspecifically. In the presence of Ca^{++}, PFP polymerizes into supramolecular tubules with molecular mass 10^6 daltons on the cell membrane and thus cause cell death. These tubules resist dissociation by SDS and reducing agents (Young et al., 1986).

What triggers the secretion of PFP is not yet clear. Calcium ionophores stimulate degranulation of CTL and NK cells may indicate that increase of cytosolic Ca^{++} levels may trigger secretion. A localized release of PFP into the contact zone may contribute to the formation of a tightly sealed and diffusion-limited microenvironment. However, how the CTL and NK cells can avoid self-lysis remains obscure. It has been proposed that a surface protein (protectin?) which can neutralize PFP rapidly may be present on the effector cell membrane (Young et al., 1988). However, since there are other lytic mechanisms involved in cell-mediated killing, other protection mechanisms may also be present.

On the other hand, there are evidence against perforin/cytolysin as a primary mechanism (Clark, 1988). Firstly, cytolytic granules, perforin or serine esterases are not detected in freshly harvested peritoneal exudate lymphocytes and no complement-like ring structures are found on lysed target cell membranes (Berke and Rosen, 1987). Only when these cells are cultured with IL-2 for 72 hrs, perforin and serine esterase can

be detected in them. Secondly, it is well known that perforin or cytolysin has an absolute requirement for Ca^{++} for assembly and function (Henkart et al., 1984; Podack et al., 1985). However,it has been found that certain target cells can be killed by primary CTL and cloned CTL in the absence of Ca^{++} (Tirosh and Berke, 1985; Ostergaard et al., 1987; Trem et al., 1987). This was also seen in lectin-mediated lysis and in CTL-mediated lysis induced by phorbol esters and calcium ionophores. Moreover, some cell lines used as sources for cytotoxic granules and perforin can kill target cells extremely well in the complete absence of Ca^{++}. Thirdly, among those antigen-stimulated, IL-2 supplemented CTL clones that have antigen specificity, only a minority contain detectable perforin but they are more or less equally cytolytic and all contain the slower acting, Ca^{++} -independent TNF-like soluble toxin activity that is probably granule-associated (Liu et al., 1987). Therefore, perforin/cytolysin may not be a central component in cell-mediated lysis and other killing mechanisms may be involved.

In addition to PFP, there are several other lytic mediators that may have a direct role in cell killing (Marx, 1986; Henkart et al; 1985; Young and Cohn, 1987). These include : 1. reactive oxygen metabolism intermediates generated by neutrophils and macrophages during oxidative injury of target cells. Moreover, hydroxyl radicals , presumably produced in the lipooxygenase pathway of fatty acid metabolism, may be involved in NK-related reactions; 2. lymphotoxin and tumour necrosis factor produced by lymphocytes and macrophages; 3. serine proteases generated in NK

cells and CTL have also been implicated in cytotoxicity; 4. the 28 kD serine esterases which are located in cytoplasmic granules of CTL and NK cells may also be released during cell killing (Pasternak and Eison, 1985); 5. leukoregulin which is a lymphokine secreted by NK cells that predominantly plays a cytostatic function on a variety of tumour cell lines; 6. leukalexin with a molecular weight of 50,000 (in reducing form) is found abundantly in the granules of CTL and can lyse a variety of targets including TNF and lymphotoxin-insensitive target cells (Liu et al., 1987). 7. NKCF which is released into the medium by mouse and human lymphocytes after contact with tumour cells or mitogens can kill a variety of NK-sensitive target cells (Bonavida, 1987). In addition, there are various killer molecules still to be defined and classified in terms of their roles of cell killing and thus will not be mentioned here (Green et al., 1986; Schmid et al., 1986; Liu et al., 1987).

It is interesting that different lytic molecules may be bound to the highly negatively charged proteoglycans that have been found in NK cell granules (MacDernott et al., 1985; Tschopp and Conzelmann, 1986) . PFP might function as transmembrane channels for other lytic toxins and enzymes. The permeation of other toxic molecules of granule origin (serine esterases, nucleases? or lymphotoxin?) through the membrane might explain the early disintegration of the target cell nucleus and fragmentation of target DNA that is observed during target cell killing by CTL (Russell, 1983; Howell and Martz, 1988).

In conclusion, our understanding on the biochemical mechanisms of lymphocyte-mediated killing is still in its infancy. Undoubtedly recent findings have suggested that this type of killing is rather complex, probably involving multiple mechanisms as well as multiple mediators. With the availability of large quantities of defined and cloned cell populations and the development of subtractive cDNA cloning techniques, it is hoped that these will eventually provide us with better insight into the molecular mechanisms underlying cell-mediated cytotoxicity.

CHAPTER TWO : MATERIALS AND METHODS

CONTENTS

I.MATERIALS

Animals

Inbred C57BL/6J($H\text{-}2^b$) mice, and guinea pigs were bred at the University Animal House, The Chinese University of Hong Kong. In each experiment, animals of the same age (8-12 weeks old) and sex were used.

Anti-Thy 1.2 Antibody

Monoclonal anti-Thy 1.2 antibody (F7D5) was purchased from Serotec Co., U.K. It was dissolved in sterile double distilled water (DDW) and stored as 0.2 ml aliquots at -70 °C before use.

Calcium Ionophore A23187

Calcium ionophore A23187 was purchased from Sigma Chem. Co., U.S.A. Stock solution was prepared as 5 mM with dimethylsulfoxide (DMSO) and stored at -20 °C as 0.1 ml aliquots. It was protected from light by wrapping the vials with tin foil.

Calf Serum

Calf serum obtained from Gibco, U.S.A. was stored at -20 °C as 10 and 20 ml aliquots.

Cell Lines

Several murine cell lines including EL-4($H\text{-}2^b$), MBL-2($H\text{-}2^b$), P815-X2($H\text{-}2^d$), WEHI-3($H\text{-}2^d$) and YAC-1 ($H\text{-}2^a$) were used in this study. All these cell lines were maintained mainly in suspension culture in RPMI medium supplemented with 10% foetal calf

serum(FCS) and containing antibiotics (100 units/ml of penicillin G, 100ug/ml of streptomycin sulfate and 3 ug/ml of fungizone).

EL-4 was obtained from the Department of Parasitology, National Institute for Medical Research, London. It is a benzopyrene-induced T cell lymphoma of C57BL/6J mice and was used for the large scale production of interleukin-2 *in vitro*.

MBL-2 tumour is a Moloney leukemia virus-induced T-cell lymphoma of C57BL/6J mice and was the major tumour model used. It was maintained *in vitro* in suspension culture and *in vivo* by weekly intraperitoneal (i.p.) passage in syngeneic C57BL/6J mice.

P815-X2 is a methylcholantrene-induced mastocytoma of DBA/2 mice and was used as a NK-insensitive tumour target in cytotoxic assay.

WEHI-3 cell line was a generous gift from Dr. A.A.Nash, Department of Pathology, University of Cambridge. It is a myelomonocytic leukemia cell line that was originally derived from a BALB/c mouse.

YAC-1 is a Moloney virus-induced T-cell lymphoma of A/Sn mice and was used as a tumour target in natural killer cell assay.

Complement

Guinea pig serum provides a source of complement. Blood of anaesthetized guinea pig was collected by cardiac puncture with a 10 ml syringe fitted with a 19G needle. Whole blood was immediately allowed to clot by standing at 4 ^{o}C for 3 hrs. Clear serum was collected without disturbing the clot. The serum was freed of red cells and debris by centrifugation. In order to

remove the autoantibodies against C57BL/6J splenocytes in serum, the serum was incubated with excess C57BL/6J splenocytes at 4 °C for 30 mins. After removal of splenocytes by centrifugation, the serum was stored at -20 °C as 1 ml aliquots. Each batch of serum was tested for complement activity before use in experiments.

Ficoll-Isopaque Solution

Ficoll 400 was purchased from Pharmacia Fine Chemicals, Sweden and metrizoic acid was obtained from Sigma Chem. Co., U.S.A. . 14 g of Ficoll 400 was dissolved in 100 ml DDW (14%, w/v) and stirred overnight. Ficoll solution was autoclaved and kept at 4 °C. 30 ml of metrizoic acid was mixed with 70 ml of Ficoll 400 solution and the mixture was protected from light by wrapping the bottle with tin foil. This solution was stored at 4 °C and warmed up to room temperature just before use.

$CaCl_2$ Solution

$CaCl_2$ was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 1 M with DDW and stored at 4 °C before use.

Cyclophosphamide

Cyclophosphamide was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 10 mg/ml with normal saline and stored at -20 °C before use.

Ethylene glycol- bis(beta-aminoethyl ether)- N,N,N',N'- tetra acetic Acid (EGTA)

EGTA was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 100 mM with DDW and stored at 4 °C before use .

Foetal Calf Serum

Foetal calf serum was obtained from Gibco, U.S.A. and was stored as 10 and 20 ml aliquots at -20 °C until use. Heat inactivated FCS (HIFCS) was prepared by incubating the aliquots at 56 °C for 30 mins. FCS was usually used as a supplement to RPMI medium for cell culture and the final concentration was normally 10% . HIFCS, on the other hand, was used as a serum supplement for cytotoxicity assays.

1-(5-Isoquinolinyl sulfonyl)-2-methylpiperazine (H7)

H7 was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 1 mM with plain RPMI medium and stored as 1 ml aliquots at -20 °C before use. It was protected from light by wrapping the vials with tin foil.

Lectins

Lectins used in the in vitro cultures include concanavalin A (Con A), phytohaemagglutinin(PHA) and succinyl-concanavalin A (sCon A). They were obtained from the Sigma Chem. Co., U.S.A.. Stock solutions (1 mg/ml) in sterile PBS were sterilized by millipore filtration and kept as 0.5 ml aliquots at -20 °C.

2-Mercaptoethanol Solution

A 0.1 M stock solution of 2-mercaptoethanol(Sigma Chem. Co., U.S.A.) was prepared with sterilized plain RPMI medium and kept at -20 °C in 0.5 ml aliquots.

α-Methyl-D-mannoside

α-methyl-D-mannoside was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 200 mg/ml with plain RPMI medium and stored at 4 °C before use.

$MgSO_4$

$MgSO_4$ was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 1 M with DDW and stored at 4 °C before use.

Mitomycin C Solution

Mitomycin C (from Streptomyces caespitosus, Sigma Chem. Co., U.S.A.) was dissolved in sterile RPMI medium with a final concentration of 0.5 mg/ml. It was protected from light by wrapping the vial with tin foil and stored at 4 °C.

Neomycin

Neomycin was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 100 mM with plain RPMI medium and was stored as 1 ml aliquots at -20 °C before use.

Nylon Wool Column

Nylon wool was a gift from Dr. D.Y. Sia, Medical Research

Council, Cambridge, U.K.. About 1 g of nylon wool was packed into a 20 ml glass syringe and then wrapped with tin foil before use. It was sterilized by autoclaving at 121 °C for 30 mins.

Penicillin-Streptomycin-Fungizone Solution

Penicillin-streptomycin-fungizone solution (PSF) was purchased from Gibco, U.S.A. and contained 10,000 units/ml of penicillin G, 10,000 ug/ml of streptomycin sulfate and 300 ug/ml of amphotericin B. This stock solution was stored at -20 °C in 5 ml aliquots. Generally, complete RPMI culture medium was supplemented with 10% FCS and 1% PSF.

Phorbol Myristate Acetate (PMA)

PMA was purchased from Sigma Chem. Co.,U.S.A.. It was dissolved in DMSO at a concentration of 5 mg/ml and stored as 0.2 ml aliquots at -20 °C before use. PMA was protected from light by wrapping the vials with tin foil.

Phosphate-buffered-saline(PBS)

PBS was prepared by dissolving 8.0 g NaCl , 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1000 ml DDW. The pH of the solution was adjusted to 7.2-7.4 and the solution was sterilized by autoclaving at 121 °C for 15 mins.

Polyinosinic-Polycytidylic Acid (poly I:C)

Poly I:C purchased from Sigma Chem. Co., U.S.A. was dissolved in PBS as 1 mg/ml and kept at 4 °C. Each mouse was injected with 0.1 ml (100 ug/mouse) intravenously (i.v.) 24 hrs

before its splenocytes were assay for cytotoxicity on tumour targets.

RPMI-1640 Medium

RPMI-1640 powder (Sigma Chem. Co., U.S.A. and Gibco, U.S.A.) for 1 litre preparation was dissolved in 1 litre of DDW and supplemented with 2.0g $NaHCO_3$ and 4.766 g Hepes(Sigma Chem. Co., U.S.A.). The medium was adjusted to pH 7.2 - 7.4 and sterilized by membrane filtration.

Scintillant

Scintillant was prepared by mixing 0.01% (w/v) dimethyl-1,4-bis(2-(5-phenyloxazol)) benzene (POPOP) and 0.4% (w/v) 2,5-diphenyloxazole (PPO) in toluene (Fisher) and was stirred overnight before use.

Sodium Chromate ($Na_2{}^{51}CrO_4$) Solution

$Na_2{}^{51}CrO_4$ solution (350-600 mCi/mg chromium) was purchased from Amersham, U.K.. Working solution was diluted to about 4 mCi/ml with sterilized RPMI medium supplemented with antibiotics. For maximum labelling, 200 µCi ^{51}Cr was added to 5 x 10^6 cells in a total volume of 0.4 ml and incubated at 37 °C for 1 hr with occassional gentle shaking.

(Methyl-^{3}H) Thymidine (^{3}H-TdR) Solution

(Methy-^{3}H) Thymidine (2 Ci/mmol) was purchased from Amersham Co., U.K.. 0.5 µCi/ul working solution was prepared by

diluting the stock solution with sterilized PBS . For pulse labelling, 0.5 µl working solution was added to each culture well.

Trypan Blue Solution

0.1%(w/v) Trypan blue (Sigma Chem. Co., U.S.A.) in PBS was used to assess the viability of cells.

N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W7)

W7 was obtained from Sigma Chem. Co., U.S.A.. Stock solution was prepared as 10 mg/ml with DMSO and stored as 0.2 ml aliquots at -20 °C before use.

II. METHODS

Cell Preparation and Isolation :

Spleen Cells

Spleens from groups of 3-4 mice were removed aseptically, minced with a pair of scissors and pressed through a sterilized 60 mesh stainless steel screen with the plunger of a plastic 5 ml syringe. Large cell clumps and debris were removed by centrifuging at 300x g for 5 seconds. The cell suspension was then spun at 300x g and washed twice with plain RPMI medium. Single cells were resuspended in complete RPMI medium and kept at 4 °C. Viability of the cell suspension was determined by the trypan blue dye exclusion method(Philip, 1973) and usually was found to be greater than 90 %.

Depletion of T Cells by Anti-Thy 1.2 Antibody and Complement Treatment

Monoclonal anti-Thy 1.2 antibody was added to spleen cell suspension (2×10^7 cells/ml) at a final dilution of 1 : 1000. The mixture was then incubated at room temperature for 30 mins. The cells were spun down and resuspended at the same concentration with appropriately diluted guinea pig complement (usually diluted 1 : 10 with serum-free medium). After 45 min incubation at 37 °C , cells were washed with plain RPMI medium for three times and then adjusted to the desired cell concentration in RPMI medium supplemented with 10% HIFCS and PSF.

T Cell Enrichment by Nylon wool Column

First, the nylon wool column was thoroughly rinsed with 50 ml prewarmed plain RPMI medium . Then the nylon wool was covered with RPMI medium supplemented with 10% HIFCS and the column was freed of air bubbles by agitation with a pipette. The nylon wool column was then incubated at 37 °C for 1 hr. Meanwhile, splenocyte suspension was prepared at a concentration of 10^8 cells/ml and prewarmed up to 37 °C . Just before adding the cell suspension, the nylon wool column was rinsed with 5-10 ml of prewarmed medium and then allowed to run dry. 2 ml of cell suspension was added and allowed to penetrate the column. An additional 0.5ml complete medium was added so as to cover the entire nylon wool column. Then the column was closed and incubated at 37 °C for 1 hr.

Nylon wool nonadherent cells (enriched for T cells) can be collected into a 50 ml centrifuge tube by washing the column with 40-50 ml prewarmed medium supplemented with 5 % HIFCS. The flow rate was 1 drop per second. The cells were spun down at 300x g for 5 mins and then resuspended in RPMI medium supplemented with 10% HIFCS

Nylon wool adherent cells (consisted of mainly B cells and macrophages) can also be collected. After removal of T cells , the nylon wool column was cooled to 4 °C and cold plain RPMI medium was then added to the column . Adherent cells can be recovered by pushing the fluid forcibly out of the column with a plunger.

Mitomycin C Treatment of Cells

Splenocytes(5×10^7 /ml) or tumour cells(5×10^6 /ml)were prepared in complete RPMI medium (RPMI + 10% FCS). Mitomycin C was added to the cell suspension at a final concentration of 50 µg/ml. The mixture was incubated for 30 mins at 37 °C with occassional shaking. Cells were washed three times with plain RPMI medium before use.

Determination of Splenocyte Cytotoxicity

Cytotoxicity assays were carried out in triplicates using 96-well flat-bottomed microtiter plates(Nunc, Denmark). The total volume of each well was 200 ul. Normal splenocytes were used as effector cells . Tumour targets were labelled with 200 uCi $Na_2\,^{51}CrO_4$ for 1 hr at 37 °C with occassional gentle shaking. The cells were then washed two times with plain RPMI medium and adjusted to 2-4 x 10^5 cells/ml in RPMI medium supplemented with 10% HIFCS and PSF. Effector:target cell ratios of 50:1 and 100:1 were always used. The assays were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After a certain period of incubation, 100 ul supernatant was carefully sucked up from each well and its radioactivity was counted in a Beckman gamma counter. For spontaneous release, 0.1 ml RPMI medium supplemented with 10% HIFCS and PSF in the absence or presence of lectins or drugs was added to 0.1 ml labelled target cells . For maximum releasable ^{51}Cr, 0.1 ml 10% triton-X100 was added to 0.1 ml labelled target cells. For the test release, effector cells were added to labelled target cells in the absence or presence of

lectins or drugs to make up to a final volume of 0.2 ml. The percentage ^{51}Cr release was calculated by the following formula:

$$\% \text{ Specific } ^{51}Cr \text{ release} = \frac{T - S}{M - S} \times 100$$

where

M : Maximum releasable counts
S : Spontaneous release counts
T : Test release counts

Ficoll-Isopaque Gradient Centrifugation

This procedure removes both red blood cells and dead cells from viable lymphocytes in a single step with high efficiency. Briefly, cell suspension and Ficoll-Isopaque solution were prewarmed to room temperature in a water bath. 4 ml of Ficoll-Isopaque solution was added to a polycarbonate tube and 2-6 ml of the cell suspension was gently layered on top of the Ficoll-Isopaque solution. The tube was centrifuged at 2000x g for 15 mins at room temperature. Viable cells were collected by harvesting the interphase cell layer. The cells were washed three times with RPMI medium before use.

In Vivo Immunization of Mice with Mitomycin C-Treated Tumour Cells

Primary immune splenocytes were obtained from C57BL/6J mice 7 days after intravenous injection with 10^7 mitomycin C-treated MBL-2 cells. Similarly, secondary immune splenocytes were obtained from C57BL/6J mice immunized twice with mitomycin C-treated MBL-2 cells. The second immunization was done at least 20 days after the first immunization and the cells were harvested

3 days after the second injection of tumour cells.

Mixed Lymphocyte Tumour Culture

Totally 1.2 x 10^8 normal or immune C57 BL/6J splenocytes were co-cultured with 6 x 10^6 mitomycin C-treated MBL-2 tumour cells in 50 ml RPMI medium supplemented with 10 % FCS and 5 x 10^{-5} M 2-mercaptoethanol (2-ME) and the cell mixtures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 5 day incubation, effector cells were harvested and dead cells were removed by Ficoll-Isopaque gradient centrifugation. The cytotoxicity of the effector cells generated was measured by the standard ^{51}Cr-release assay using MBL-2, P815 and EL-4 cells as targets. Effector:target ratios of 10:1 and 20:1 were normally used and the incubation time was 8 hrs.

Production of Interleukin-2

Large scale production of interleukin-2 (IL-2) was achieved by *in vitro* stimulation of EL-4 cells with PMA (Farrar *et al.*, 1980). 4 x 10^7 EL-4 cells were cultured with 4 µg PMA in 40 ml RPMI medium supplemented with 2% FCS and incubated at 37 °C in 5% CO_2 for 6 hrs. After two washings, the cells were resuspended in 40 ml RPMI medium containing 5% FCS and incubated for a further period of 24 hrs. Cells were then pelleted by centrifugation at 300x g for 5 mins and the supernatant was harvested and stored at -20 °C as 10 and 20 ml aliquots. IL-2 activity of the supernatant was measured by using the IL-2 assay as described by Lafferty *et al.* (1980).

Proliferative Response of In Vitro Cultured Lymphocytes

After 5 days of *in vitro* mixed lymphocyte tumour culture, a portion of the viable cells was collected and further cultured in RPMI medium supplemented with 10% FCS and 5×10^{-5} M 2-ME for 2 days. Then the cells were harvested and dead cells were removed by Ficoll-Isopaque gradient centrifugation. The cell suspensions were adjusted to a final concentration of 10^6 cells/ml. Mitomycin C-treated tumour cells were used as stimulators and adjusted to a concentration of 2×10^5 cells/ml. 50 ul cells and 50 ul stimulators were added to each well of a 96-well microtiter plate. 100 ul complete RPMI medium supplemented with 5×10^{-5} M 2-ME and containing no IL-2 or 10% IL-2 was added to each well. The stimulators used included the MBL-2, P815 and EL-4 cells. After 48 hr incubation at 37°C in a humidified atmosphere of 5 % CO_2 in air , the cultures were pulsed with 0.5 μCi ^{3}H-TdR for 16 hrs before harvesting onto glass fibre filter using a Titertek multi-harvester (Flow Lab. Ltd.,U.K.). Incorporated ^{3}H-TdR label was counted in a Beckman scintillation counter (Beckman LS1801, U.S.A.) and the results were expressed as counts per minute (CPM).

Statistical Analysis

All results were expressed as the arithmetic mean ± standard error (S.E.). Student's 't'test was used to determine the confidence limits in group comparison. Normally $p <= 0.05$ was regarded as significant difference.

CHAPTER THREE : INDUCTION OF NONSPECIFIC LYMPHOCYTE-MEDIATED CYTOTOXICITY AGAINST TUMOURS BY LECTINS

CONTENTS

INTRODUCTION

The study of lectin-dependent cell-mediated cytotoxicity (LDCC) was initially based on the observation of Moller (1965). She and others (Rubens and Henney, 1977; Thierry et al., 1977; Tartof and Fitch, 1978; Waterfield et al., 1981; Eugene et al., 1982) have found that alloimmune spleen cell populations and concanavalin A (Con A) or phytohemagglutinin (PHA)-activated splenocytes could kill a wide spectrum of tumour targets, including those of syngeneic origin, when incubated with Con A or PHA at the same time. In addition, it was demonstrated that specific cloned cytotoxic T lymphocytes and even cloned helper T cells could lyse syngeneic and third party target cells efficiently in the presence of lectins (Bonavida et al., 1983; Leung and Nash, 1987). Moreover, some investigators reported that polymorphonuclear leukocytes were also capable of lysing various tumour cells in the presence of plant lectins (Clark and Klebanoff, 1979) or animal lectins (Yamazaki et al., 1983). In contrast, Tartof (1980) showed that increasing concentrations of Con A could inhibit the immunologically specific cytotoxic T lymphocyte-mediated cytolysis. However, all these investigators did not observe any significant cytolysis of syngeneic or allogeneic target cells in the presence of lectins when normal, rather than alloimmune or mitogen-activated spleen cells were used as the effector cells.

Current work in our laboratory has been focussed on the *in vitro* activation and modulation of lymphocyte-mediated cytotoxicity against syngeneic tumours. In contrast to the previous reports, the present investigation has shown that under appropriate conditions, various mitogenic lectins (including PHA, Con A and its succinylated derivative) were found to be capable of inducing normal mouse splenocytes to lyse various tumour targets. In addition, this lectin-induced cytolysis of tumour targets was found to be T cell-mediated and calcium- and temperature-dependent.

RESULTS

3.1 Dose response of lectin-induced lymphocyte-mediated cytotoxicity against tumours :

In order to determine whether mitogenic lectins can induce normal splenocytes to lyse various tumour targets *in vitro*, three plant lectins (Con A, succinyl Con A, PHA) were chosen for the present study. Normal splenocytes from C57 BL/6J mice ($H\text{-}2^b$) were incubated with ^{51}Cr-labelled allogeneic YAC-1 cells ($H\text{-}2^a$) or similarly labelled syngeneic MBL-2 cells ($H\text{-}2^b$) for 6 and 10 hrs respectively in the presence of various concentrations of lectins and the specific lysis of the these two tumour targets was determined.

As shown in Fig. 3.1, it can be seen that Con A could induce significant splenocyte cytotoxicity against the two tumour targets in a dose-dependent manner. The optimal concentration of Con A for the induction of LDCC was found to be 3-6 ug/ml. At higher concentrations Con A exhibited diminished ability on the induction of LDCC, so Con A at a concentration of 6 µg/ml was chosen for subsequent experiments. In contrast, sCon A did not show diminished ability on induction of LDCC at higher concentrations. In fact, sCon A induced splenocyte cytotoxicity increased with increasing concentrations of sCon A up to a concentration of 12 µg/ml. Therefore, for direct comparison to the LDCC induced by that of Con A, sCon A at a concentration of 6 µg/ml was deliberately chosen for further studies.

Similarly, PHA was also found to be effective in mediating LDCC although it was much less potent than that of Con A and sCon A (Fig. 3.2). The optimal concentration of PHA for the induction of LDCC were found to be 2.5 µg/ml for YAC-1 target and 10 µg/ml for MBL-2 target.

It should be noted that the spontaneous ^{51}Cr release from the two tumour targets was not significantly different in the absence and in the presence of lectins at all concentrations used in our experiments in a 6 hr (YAC-1) or 10 hr (MBL-2) ^{51}Cr-release assay (data not shown). Thus, the three plant lectins, when present alone, are not toxic to the two tumour targets under the prescribed experimental conditions. Since Con A and sCon A are much more potent than PHA for the induction of LDCC against the two tumour targets, these two lectins will be primarily used in further studies.

3.2 Time course of lectin-induced lymphocyte-mediated cytotoxicity against tumours

In order to determine the best incubation time for inducing maximum lysis of the two tumour targets, normal splenocytes from C57BL/6J were incubated with ^{51}Cr-labelled tumour targets (YAC-1 and MBL-2 cells) in the absence or presence of Con A and sCon A (6 µg/ml) for various time periods (2-12 hrs). It was found that the percentage of lectin-dependent cytolysis increased

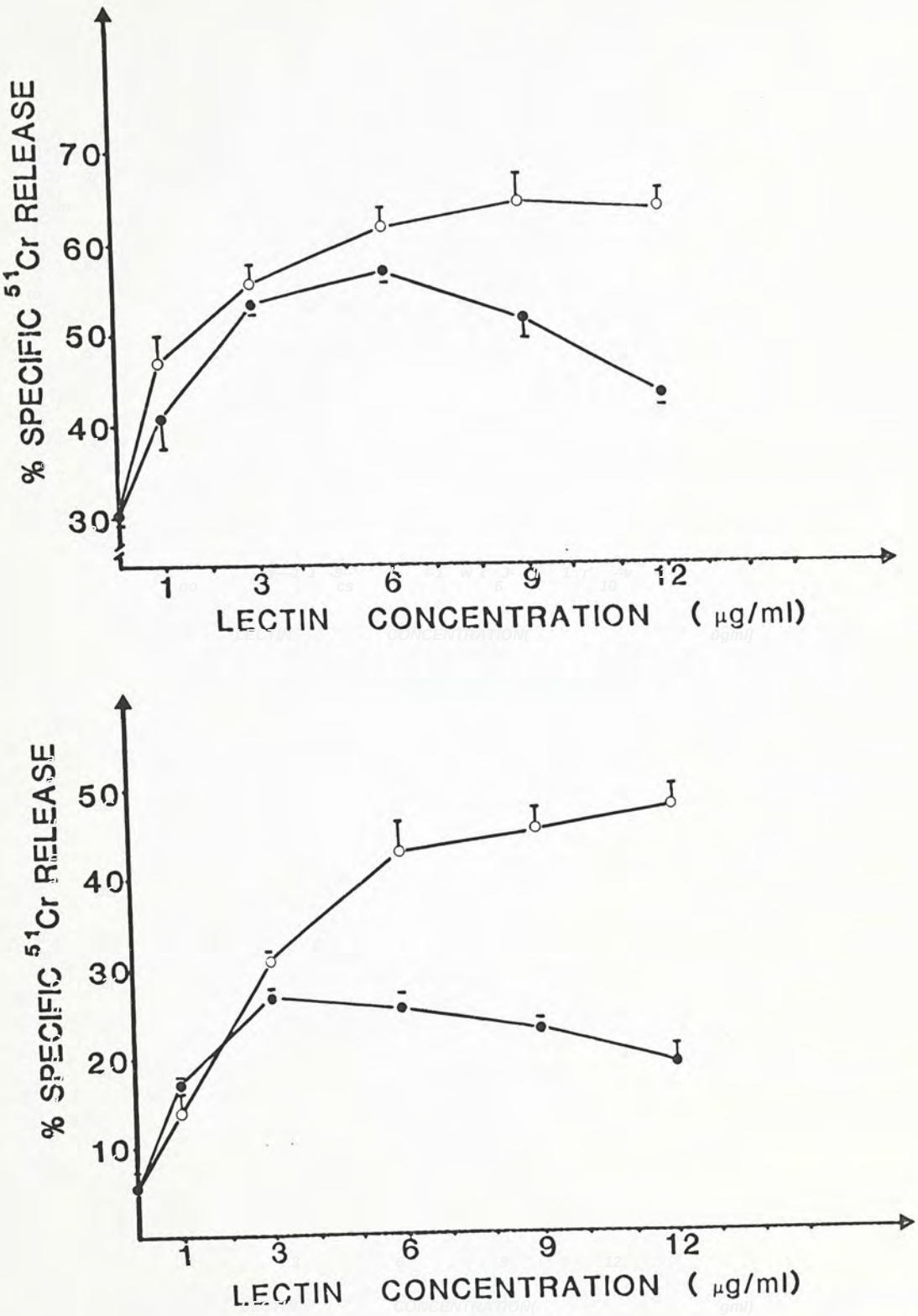

Figure 3.1 Dose response of Con A-induced lymphocyte-mediated cytotoxicity on YAC-1 and MBL-2 targets as compared to that induced by succinyl Con A (sCon A). Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1(a) and MBL-2(b) tumour target cells at an E:T ratio of 100:1 in the presence of various concentrations of Con A (●-●) or sCon A (O-O) under identical conditions in a 6 hr (a) and a 10 hr (b) ^{51}Cr-release assay.

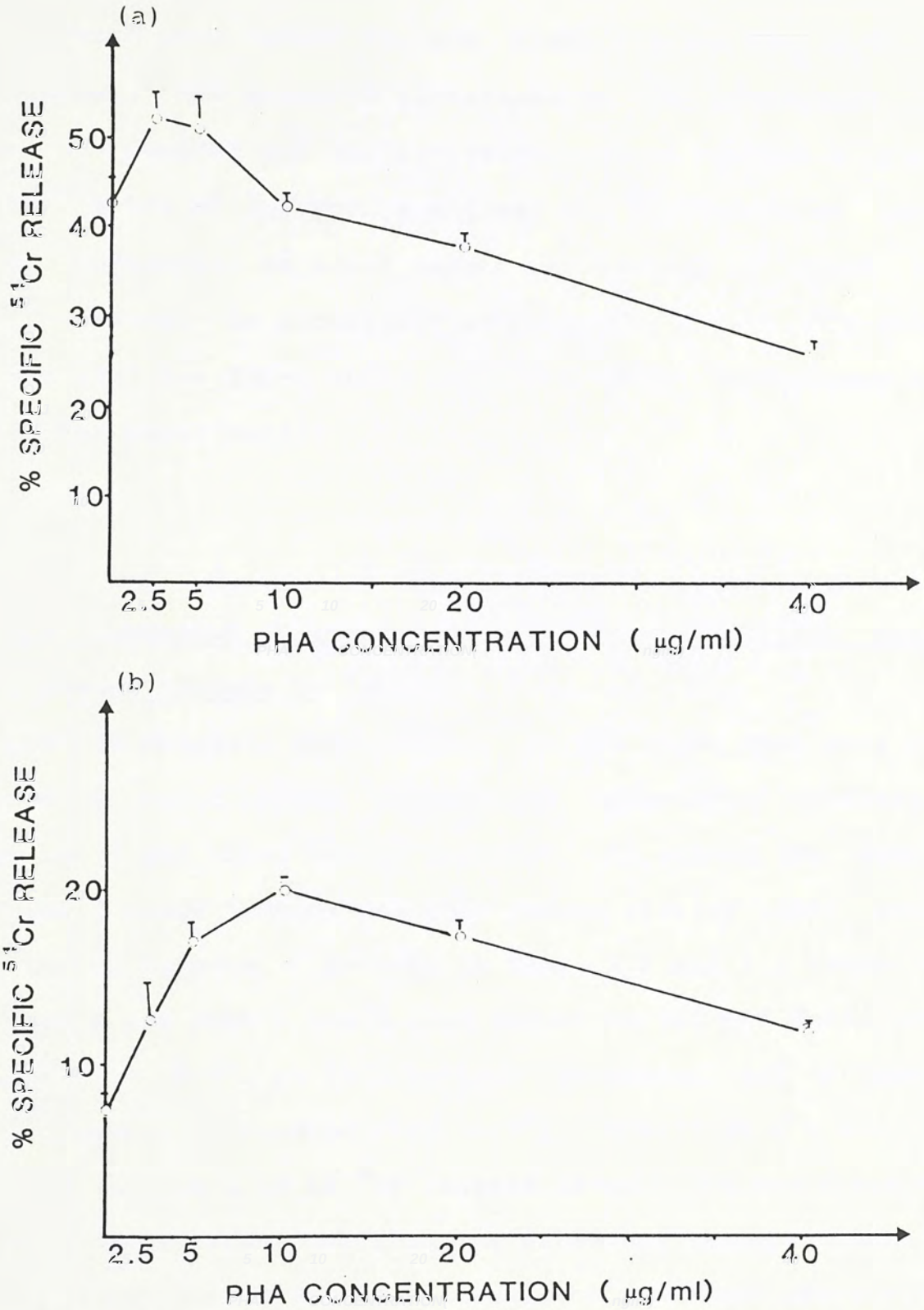

Figure 3.2 Dose response of PHA-induced lymphocyte-mediated cytotoxicity against tumours. Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) tumour target cells at an E:T ratio of 100:1 in the presence of the various concentrations of PHA in a 6 hr (a) and 10 hr (b) ^{51}Cr-release assay.

with time with both tumour targets (Fig. 3.3 and Fig. 3.4). With YAC-1 target, the greatest difference in the percentage lysis between the control and lectin-treated groups reached a plateau at about 6 hrs of incubation whereas the percentage of lectin-dependent cytolysis on MBL-2 target was maximal at about 10-12 hrs. Therefore, in subsequent experiments, a 6 hr ^{51}Cr-release assay was used for YAC-1 cells whereas a 10 hr ^{51}Cr-release assay was used for MBL-2 cells.

3.3 The induction of lymphocyte-mediated cytotoxicity against various tumour targets by lectins

From the previous experiments, it was shown that both Con A and sCon A could induce significant splenocyte cytotoxicity against YAC-1 and MBL-2 tumour targets. It raises the question as whether these lectins can also induce similar LDCC against other tumour targets. Results in Table 3.1 and 3.2 showed that both Con A and sCon A could also induce normal C57 BL/6J mouse splenocytes ($H\text{-}2^b$) to kill various syngeneic and allogeneic tumour targets, including P815 ($H\text{-}2^d$), WEHI-3 ($H\text{-}2^d$) and EL-4 ($H\text{-}2^b$) cells, in a 10 hr ^{51}Cr release assay. It was found that different tumour target cells showed different susceptibility to lectin-induced cytolysis. In general, the YAC-1 and MBL-2 tumour cells were found to be good targets for LDCC whereas the P815 and WEHI-3 cells were of intermediate sensitivity to LDCC. The EL-4 cells, on the other hand, were found to be a poor target for the lectin-induced cytolysis.

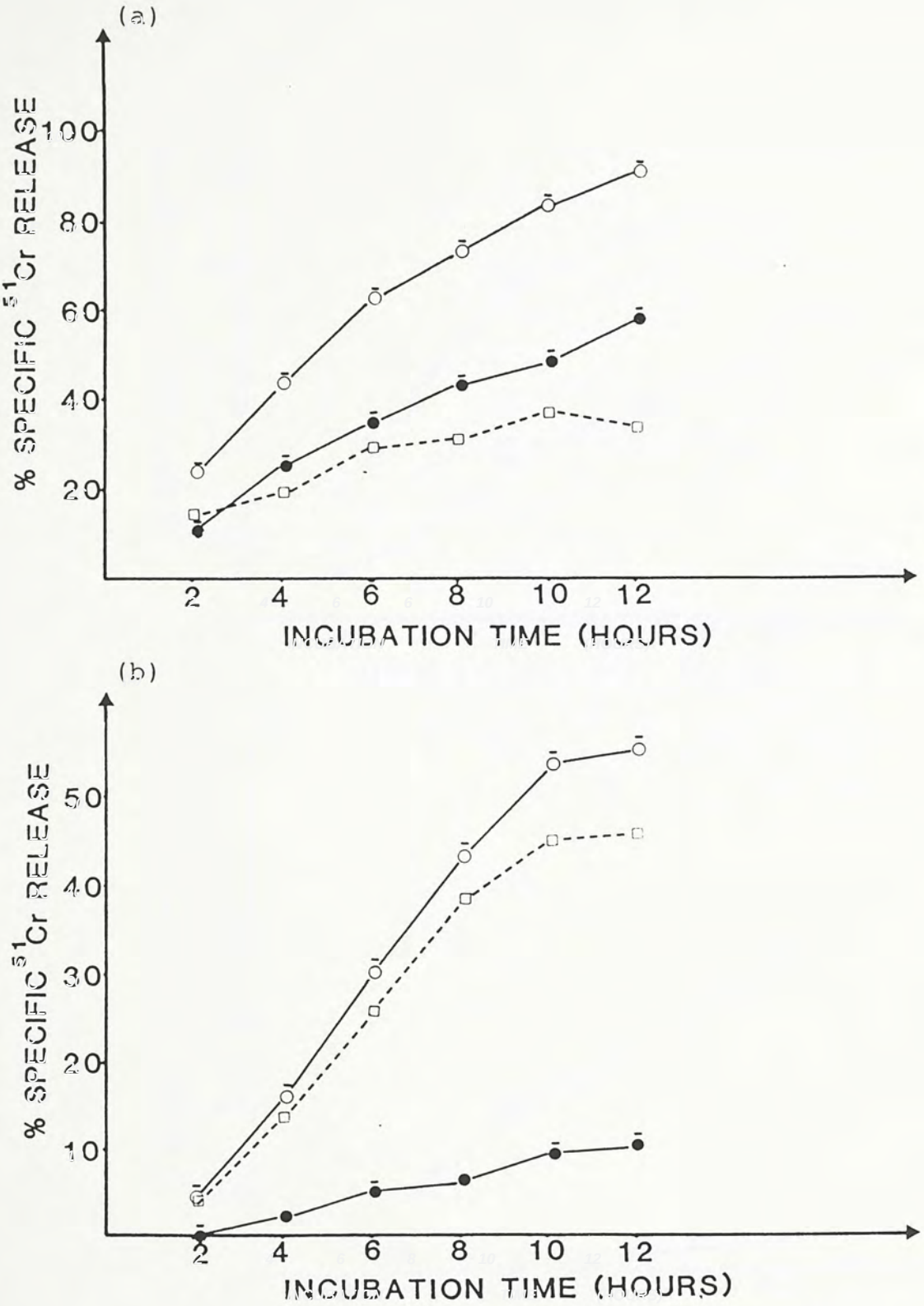

Figure 3.3 Time course of Con A-induced lymphocyte-mediated cytotoxicity against tumours. Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1(a) and MBL-2(b) tumour target cells at an E:T ratio of 100:1 in the absence (●-●) or presence (○-○) of Con A (6 µg/ml). The percentage lysis was determined every 2 hrs for 12 hrs. The difference between the percentage lysis of tumour target in the presence and absence of Con A was referred to as the percentage of Con A-dependent cytolysis (□-□).

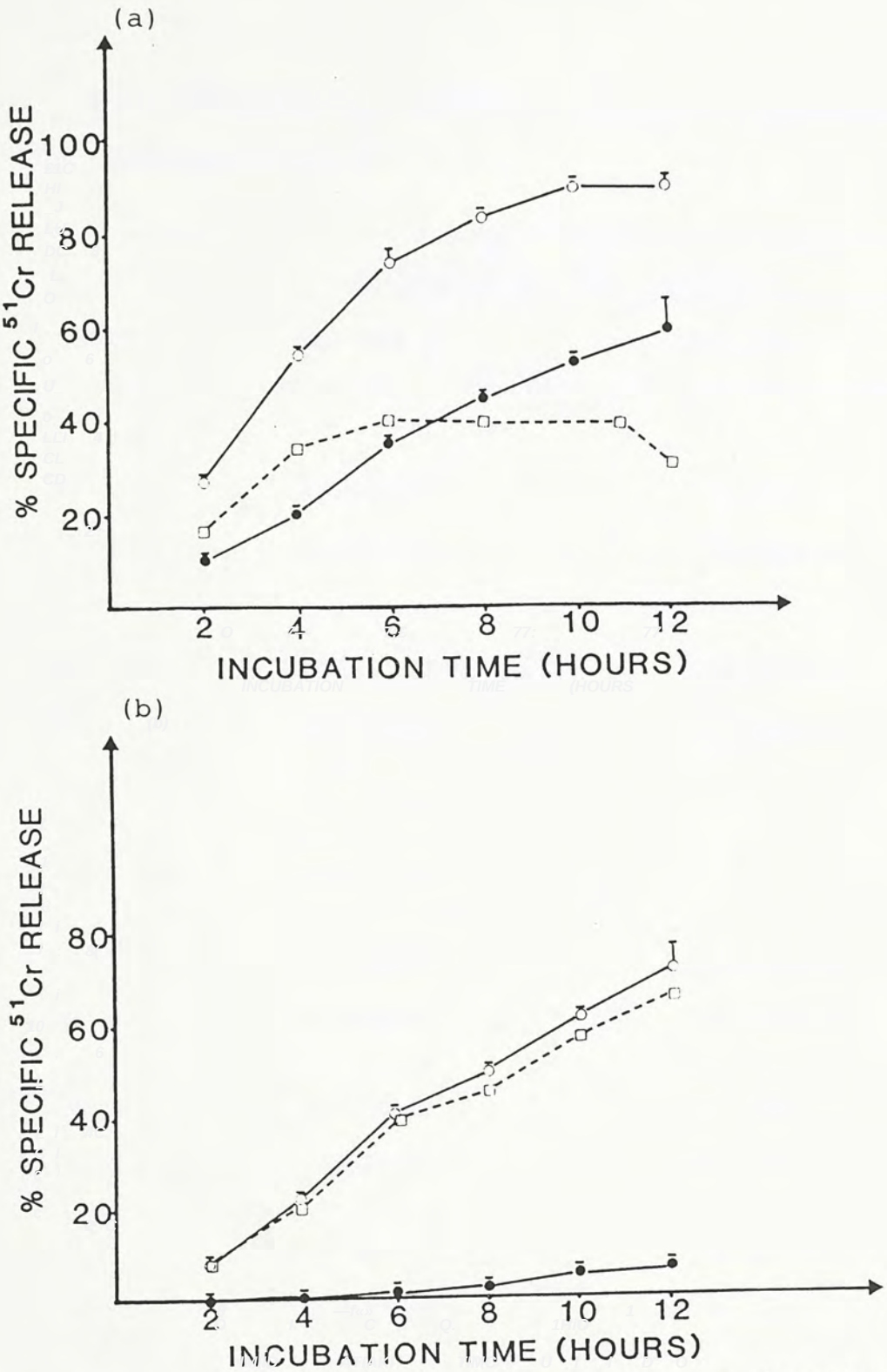

Figure 3.4 Time course of succinyl Con A-induced lymphocyte-medidated cytotoxicity against tumours. Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) tumour target cells at an E:T ratio of 100:1 in the absence (●-●) or presence (O-O) of sCon A (6 µg/ml). The percentage lysis was determined every 2 hrs for 12 hrs. The difference between the percentage lysis of tumour target in the presence and absence of sCon A was referred to as the precentage of sCon A-dependent cytolysis (□-□).

Table 3.1 The induction of lymphocyte-mediated cytotoxicity against various tumours by Con A*

Tumour target	E:T@	% Specific ^{51}Cr release	
		No Con A	With Con A
Expt 1. :			
YAC-1	50:1	27.6±1.5	51.5±1.0
	100:1	42.8±1.1	74.6±0.9
MBL-2	50:1	11.1±1.3	38.8±0.5
	100:1	12.9±1.3	58.5±0.2
Expt. 2 :			
P815	50:1	0.9±0.7	18.1±0.3
	100:1	2.4±0.5	24.6±1.3
WEHI-3	50:1	0.9±0.2	12.7±1.8
	100:1	2.1±0.7	24.4±1.5
EL-4	50:1	0	6.6±1.2
	100:1	0.2±1.4	8.3±1.7

* Normal C57 BL/6J mouse splenocytes (effector cells) were incubated with ^{51}Cr labelled tumour target cells in the absence or presence of Con A (6 µg/ml) for 10 hrs.

@ Effector-to-target cell ratio.

Table 3.2 The induction of lymphocyte-mediated cytotoxicity against various tumours by sCon A*

Tumour target	E:T@	% Specific ^{51}Cr release	
		No sCon A	With sCon A
MBL-2	50:1	6.9±1.0	31.2±1.4
	100:1	6.3±1.1	38.2±0.5
WEHI-3	50:1	0	4.5±0.3
	100:1	0.6±0.7	14.9±1.2
EL-4	50:1	0	8.4±1.4
	100:1	0.2±1.4	12.2±3.2

* Normal mouse splenocytes (effector cells) were incubated with ^{51}Cr labelled tumour target cells in the absence or presence of sCon A (6 µg/ml) for 10 hrs.

@ Effector-to-target cell ratio.

3.4 Time course of Con A-induced lymphocyte-mediated cytotoxicity against tumours : Comparison of normal and poly I:C activated splenocytes

In order to study the nature of effector cell populations activated by lectins, I have compared the normal resting lymphocytes versus the poly I:C-activated lymphocytes as a source of effector cells for LDCC. In this experiment, each normal C57 BL/6J mouse was injected intravenously (i.v.) with 100 ug poly I:C whereas the control mice received an intravenous injection of an equal volume of PBS. After 16-24 hrs, splenocytes in these two groups of mice were prepared and incubated with ^{51}Cr-labelled tumour target cells in the absence or presence of an optimal concentration of Con A (6 µg/ml) for various time periods.

As shown in Fig. 3.5, the poly I:C-activated splenocytes displayed slightly higher killing activity on the two tumour targets as compared to normal resting splenocytes in the absence of Con A. However, in the presence of Con A, both the kinetic curves and the percentage of cytotoxicity were very much similar for poly I:C-activated and normal splenocytes.

3.5 Induction of lymphocyte-mediated cytotoxicity against tumours by Con A : Comparison of normal and MMC-treated splenocytes

In order to determine whether active DNA synthesis in the

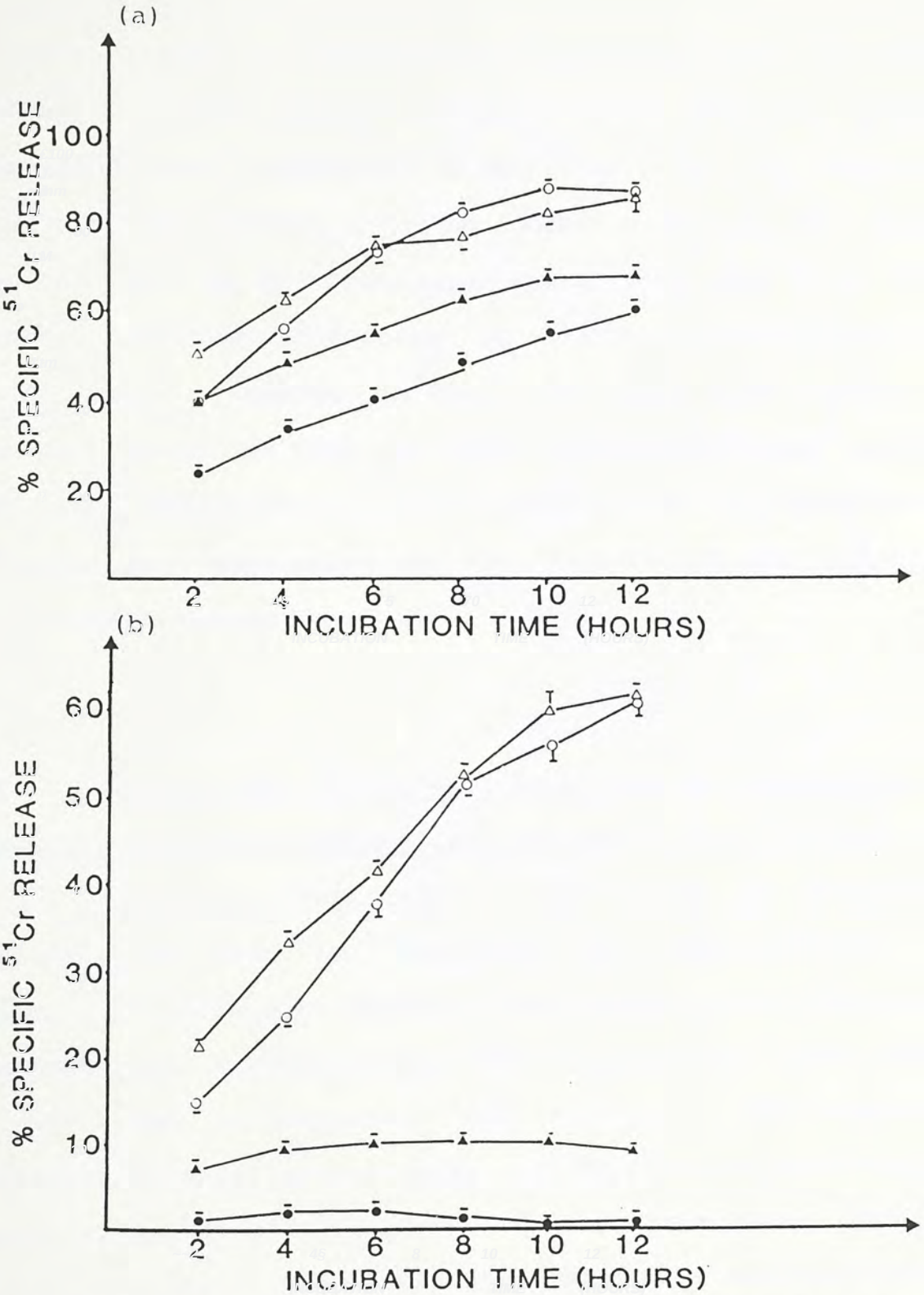

Figure 3.5 Time course of Con A-induced lymphocyte-mediated cytotoxicity against tumours : Comparison of normal and poly I:C-activated splenocytes. Normal C57 BL/6J mice were injected i.v. with 100 ug poly I:C /mouse. After 16-24 hrs, poly I:C-activated splenocytes were prepared and incubated with ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) target cells at an E:T ratio of 100:1 in the absence (▲-▲) or presence (Δ-Δ) of Con A (6 µg/ml)) for various time periods. As a control, normal splenocytes from mice injected with PBS were incubated with ^{51}Cr-labelled tumour target cells at an E:T ratio of 100:1 in the absence (●-●) or presence (○-○) of Con A under identical conditions.

effector cells is required for mediating LDCC, normal mouse splenocytes were either incubated with complete medium or pretreated with mitomycin C (MMC) for 1 hr as described in Materials and Methods so as to inhibit cellular DNA synthesis. The untreated or MMC-pretreated splenocytes were then incubated with ^{51}Cr-labelled YAC-1 target at various concentrations of Con A for 6 hrs. Results in Fig. 3.6 showed that similar dose-response curve of LDCC was given by MMC-pretreated splenocytes and normal splenocytes. This suggested that proliferation of the effector cell population was not required for the induction of LDCC against tumours by Con A.

3.6. Determination of the cell type that is responsible for the induction of lymphocyte-mediated cytotoxicity by lectins

In order to characterize the nature of effector cell population(s) that is responsible for mediating LDCC against tumours, T cells were depleted from normal mouse splenocytes by treatment with monoclonal anti-Thy 1.2 antibody plus complement as described in Materials and Methods. The untreated or complement-treated groups served as the positive controls. These three groups of effector cells were incubated with ^{51}Cr-labelled tumour targets in the absence or presence of lectins (Con A , sCon A) for the indicated periods of time. Data from Table 3.3 and 3.4 showed that 1. complement treatment alone did not affect LDCC against the two tumour targets; 2. depletion of T cells by treatment with anti-Thy 1.2 and complement completely abrogated LDCC against the tumours. These results indicate that the

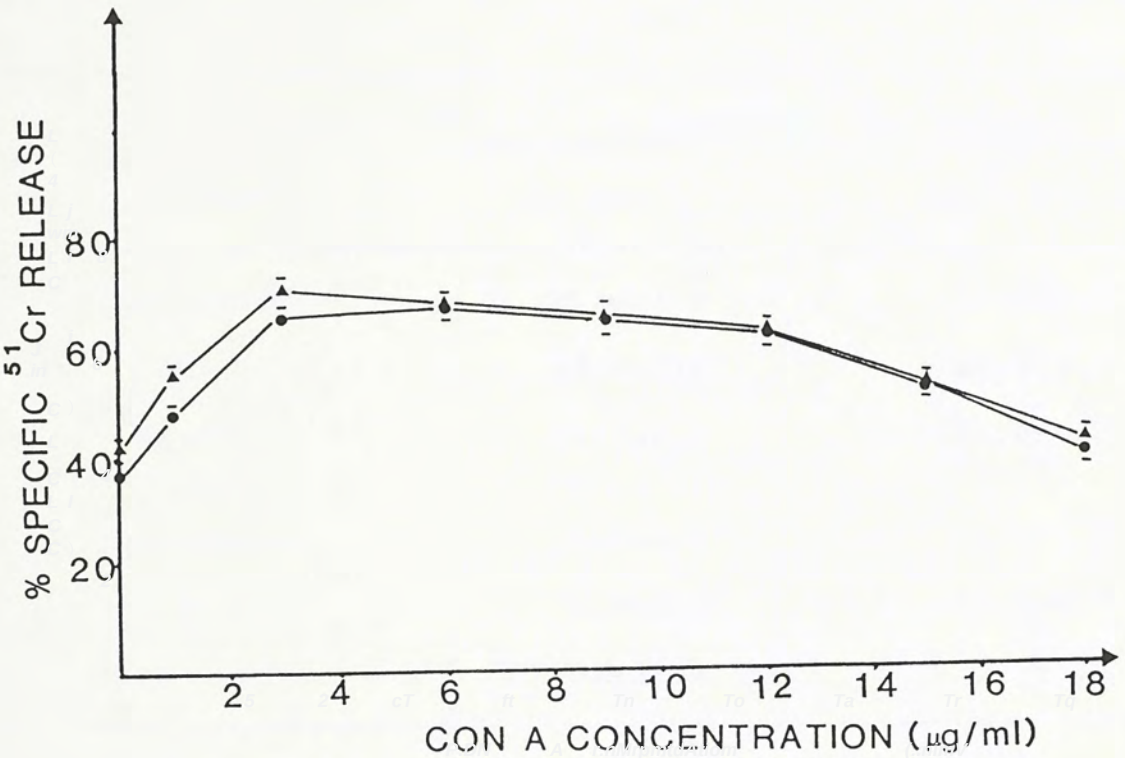

Figure 3.6 Induction of lymphocyte-mediated cytotoxicity against YAC-1 target by Con A : Comparison of normal and MMC-pretreated splenocytes. Normal mouse splenocytes were pretreated with mitomycin C for 1 hr as described in Materials and Methods. Normal untreated (●-●) or MMC-pretreated (▲-▲) splenocytes were incubated with ^{51}Cr-labelled YAC-1 tumour target cells at various concentrations of Con A at an E:T ratio of 100:1 in a 6 hr ^{51}Cr-release assay.

Table 3.3 Determination of the cell type that is responsible for the induction of lymphocyte-mediated cytotoxicity by Con A

Tumour target	Group*	% Specific ^{51}Cr release	
		No Con A	With Con A
YAC-1	untreated	25.6±1.0	63.9±1.6
	C'	27.6±2.0	65.4±3.1
	Ab+C'	24.7±0.1	23.0±1.8
MBL-2	untreated	2.9±0.5	41.0±1.9
	C'	2.0±1.2	42.6+0.7
	Ab + C'	0.4±1.2	2.7±0.6

* Normal mouse splenocytes were treated with complement only (C') or with anti-Thy 1.2 antibody plus complement (Ab +C') as described in Materials and Methods. The C', Ab + C' and untreated groups were incubated with ^{51}Cr labelled tumour target cells in the absence or presence of Con A (6 µg/ml) for 6 hrs (YAC-1) or 10 hrs (MBL-2). The effector-to-target cell ratio was 100:1.

Table 3.4 Determination of the cell type that is responsible for the induction of lymphocyte-mediated cytotoxicity by sCon A

Tumour target	Group*	E:T@	% Specific ^{51}Cr release	
			No sCon A	With sCon A
YAC-1	untreated	50:1	17.4+0.9	57.7+1.6
		100:1	31.1+1.1	71.6+0.8
	C'	50:1	16.6+2.1	51.6+1.5
		100:1	26.1+1.3	67.6+1.6
	Ab+C'	50:1	14.5+0.1	13.5+0.6
		100:1	21.6+1.3	21.1+2.3
MBL-2	untreated	50:1	6.9+1.0	31.2+1.4
		100:1	6.3+1.1	38.2+0.5
	C'	50:1	6.3+0.6	31.1+1.4
		100:1	4.7+1.2	40.9+1.1
	Ab+C'	50:1	5.4+1.0	7.8+1.1
		100:1	6.3+0.7	7.2+1.6

* Normal splenocytes treated with complement only (C') or with anti-Thy 1.2 antibody plus complement (Ab+C') as described in Materials and Methods. The C' , Ab + C' and untreated groups were then incubated with ^{51}Cr labelled tumour target cells in the absence or presence of sCon A (6µg/ml) for 6 hrs (YAC-1) or 10 hrs (MBL-2).

@ Effector-to-target cell ratio.

lectin-induced cytotoxicity is T cell-dependent.

3.7 Effect of T cell enrichment by nylon wool column on lectin-induced lymphocyte-mediated cytotoxicity

To further establish the nature of effector cells involved in LDCC, normal mouse splenocytes can be enriched for T cells by fractionation on nylon wool column. It has been known that T cells (and some null cells) can pass through nylon wool column while B cells and macrophages are preferentially retained (Trizio and Cudkowicz, 1974). In this experiment, normal mouse splenocytes were separated on nylon wool column into nonadherent and adherent fractions while unfractionated cells served as the control. As shown in Table 3.5 and 3.6, it can be seen that 1. the nylon wool nonadherent cells displayed higher killing activity against MBL-2 tumour target as compared to the control unfractionated cells in the presence of lectins; 2. the lectin-dependent cytolysis of MBL-2 tumour target was not seen with the nylon wool adherent cells. These results, when taken together, strongly indicate that the main effector cells for LDCC are T cells.

3.8 Effect of pretreatment of effector cells and/or target cells with lectins on the induction of lymphocyte-mediated cytotoxicity against tumours

Con A is a lectin which can bind oligosaccharide residues on

Table 3.5 Effect of T cell enrichment by nylon wool column on Con A-induced lymphocyte-mediated cytotoxicity against MBL-2 tumour target

Tumour target	Group*	E:T@	% Specific ^{51}Cr release	
			No Con A	With Con A
MBL-2	UNF	50:1	4.0±0.5	15.7±1.4
		100:1	4.3±0.5	20.4±0.1
	NAF	50:1	3.5±0.4	24.1±1.0
		100:1	4.8±1.0	31.9±0.8
	ADF	50:1	4.0±0.0	5.2±0.6
		100:1	7.8±0.9	5.0±0.5

* Normal mouse splenocytes were separated into two cell fractions on nylon wool column. The nonadherent fraction (NAF) was consisted of mainly T cells whereas the adherent fraction (ADF) was consisted of mainly B cells and macrophages. The unfractionated cells served as a control. These three cell fractions were incubated with ^{51}Cr-labelled MBL-2 tumour cells for 10 hrs in the absence or presence of Con A (6 µg/ml).

@ Effector-to-target cell ratio.

Table 3.6 Effect of T cell enrichment by nylon wool column on succinyl Con A-induced lymphocyte-mediated cytotoxicity against MBL-2 tumour target

Tumour target	Group*	E:T@	% Specific ^{51}Cr release	
			No sCon A	With sCon A
MBL-2	UNF	50:1	4.0±0.5	34.8±0.2
		100:1	4.3±0.5	39.9±0.9
	NAF	50:1	3.5±0.4	44.8±0.3
		100:1	4.8±1.0	53.2±0.8
	ADF	50:1	4.0±0.0	15.6±1.0
		100:1	7.8±0.9	20.4±1.2

* Normal mouse splenocytes were separated into two cell fractions on nylon wool column. The nonadherent fraction (NAF) was consisted of mainly T cells whereas the adherent fraction (ADF) was consisted of mainly B cells and macrophages. The unfractionated cells served as a control. These three fractions were incubated with ^{51}Cr labelled MBL-2 tumour cells for 10 hrs in the absence or presence of sCon A (6 μg/ml).

@ Effector-to-target cell ratio.

the glycoproteins on the surface of cells. It is of interest to know whether Con A can act either on the effector cells or target cells alone in order to induce LDCC. Therefore, normal mouse splenocytes and tumour target cells were pretreated with an optimal concentration of Con A (6 ug/ml) for 1 hr at 37°C and then washed 3 times with plain RPMI medium so as to remove the excess unbound Con A. As shown in Fig. 3.7(a), Con A pretreatment of either effector cells or YAC-1 target cells alone induced a significant but slightly lower level of LDCC as compared to that of the continuous presence of Con A in the medium. However, when Con A pretreated effector cells and target cells were mixed together, it was found that cytolysis of YAC-1 target was similar to that of the continuous presence of Con A in the medium. In contrast, Con A pretreatment of effector cells alone induced a substantial lower level of cytolysis against MBL-2 target cells when compared to Con A pretreatment of MBL-2 target cells alone (Fig. 3.7(b)). Moreover, the combination of two resulted in cytolysis against MBL-2 target cells which was similar when target cells were pretreated with Con A alone. These findings would seem to indicate that Con A could bind to either effector cells or target cells alone to induce cytolysis but the action of Con A on effector cells was different on different tumour targets.

In contrast to the previous results of Con A-induced lymphocyte-mediated cytotoxicity, it was found that pretreatment of effector cells and/or target cells with sCon A (6 µg/ml) for 1 hr failed to induce significant cytotoxicity against both tumour

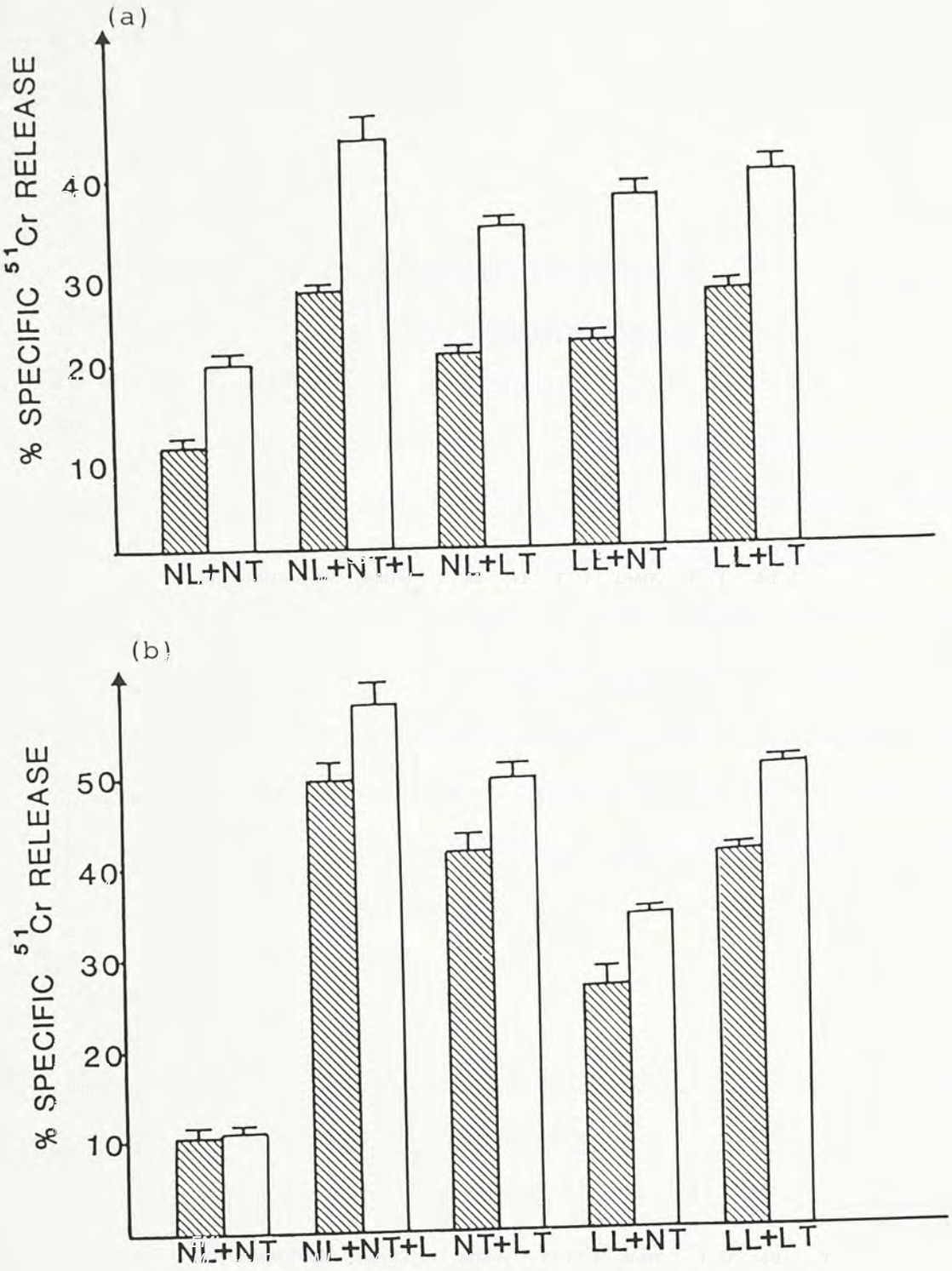

Figure 3.7 Effect of pretreatment of effector cells and/or target cells with Con A on the induction of lymphocyte-mediated cytotoxicity against tumours. Normal mouse splenocytes and tumour target cells were pretreated with Con A (6 µg/ml) for 1 hr at 37 °C. Con A-pretreated (LL) or normal (NL) splenocytes were incubated with Con A-pretreated (LT) or normal (NT) ^{51}Cr-labelled tumour target cells at a 50:1 (hatched bar) or 100:1 (open bar) E:T ratio in a 6 hr ^{51}Cr-release assay for YAC-1 target (a) and a 10 hr ^{51}Cr-release assay for MBL-2 target (b). The control groups were untreated splenocytes incubated with untreated ^{51}Cr-labelled tumour target cells in the absence or presence of Con A (6 ug/ml)(L) under identical conditions.

targets (YAC-1 and MBL-2) (Fig. 3.8).

3.9. The effect of temperature on the induction of lymphocyte-mediated cytotoxicity against tumours by lectins

In order to study the temperature requirement for induction of efficient lysis of the targets, normal mouse splenocytes were incubated with ^{51}Cr-labelled tumour target cells in the absence or presence of lectins at 37°C, room temperature (22°C) or 4°C for 6 hrs (YAC-1 tumour target) and 10 hrs (MBL-2 tumour target). Results in Table 3.7 and 3.8 showed that by cooling the cell mixture to 4°C or by maintaining the effector and target cells at room temperature throughout the incubation period, the induction of lymphocyte-mediated cytotoxicity against tumours by the two lectins was completely inhibited.

3.10 Inhibition of lectin-induced lymphocyte-mediated cytotoxicity by α-methyl-D-mannoside

α-methyl-D-mannoside (αmMan) and other α-D-glucosamine and mannopyranosides are the most effective oligosaccharide inhibitors of Con A (Baenziger and Fiete, 1979) and these sugars can rapidly bind to Con A and remove it from the cell surface. In order to determine whether the binding of the lectins (Con A, sCon A) to the cell surface is essential for 'triggering' LDCC, the inhibitory effect of α-methyl-D-mannoside on the lectin-induced lymphocyte-mediated cytotoxicity was examined . Normal

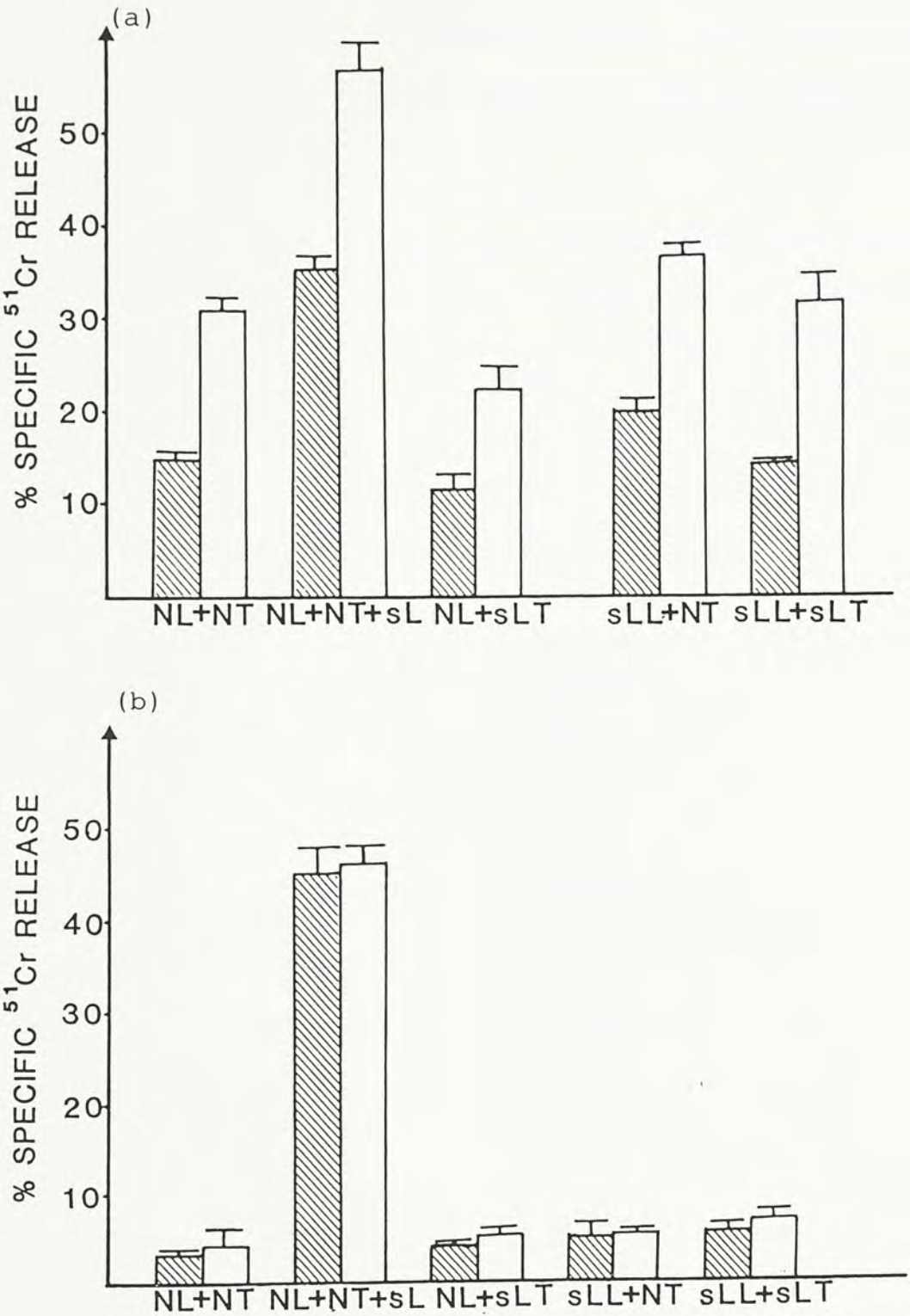

Figure 3.8 Effect of pretreatment of effector cells and/or target cells with succinyl Con A on the induction of lymphocyte-mediated cytotoxicity against tumours. Normal mouse splenocytes and tumour target cells were pretreated with sCon A (6 ug/ml) for 1 hr at 37°C. sCon A-pretreated (sLL) or normal (NL) splenocytes were incubated with sCon A-pretreated (sLT) or normal (NT) ^{51}Cr-labelled tumour target cells at a 50:1 (hatched bar) or 100:1 (open bar) E:T ratio in a 6 hr ^{51}Cr-release assay for YAC-1 target (a) and a 10 hr ^{51}Cr-release assay for MBL-2 target (b). The control groups were untreated normal splenocytes incubated with untreated ^{51}Cr-labelled tumour target cells in the absence or presence of sCon A (6 µg/ml) (sL) under identical conditions.

Table 3.7 The effect of temperature on the induction of lymphocyte-mediated cytotoxicity against tumours by Con A

Tumour target	Incubation temperature*	E:T@	% Specific ^{51}Cr release	
			No Con A	With Con A
YAC-1	37 °C	50:1	15.1±1.2	37.9±3.6
		100:1	23.7±0.5	55.8±1.6
	22 °C	50:1	1.1±0.6	2.0±0.3
		100:1	2.2±0.5	3.4±0.5
	4 °C	50:1	1.3±0.5	0.0±0.2
		100:1	0.9±0.9	0
MBL-2	37 °C	50:1	4.4±0.9	17.3±0.9
		100:1	3.4±2.3	25.6±1.2
	22 °C	50:1	0.8±0.3	1.4±0.6
		100:1	1.5±0.5	0.7±0.6
	4 °C	50:1	2.0±0.4	0.2±0.5
		100:1	2.0±0.5	0.7±0.6

* Normal mouse splenocytes were incubated with ^{51}Cr labelled tumour target cells in the absence or presence of Con A (6µg/ml) for 6 hrs (YAC-1 target) and 10 hrs (MBL-2 target) at the indicated temperatures in a air-tight box containing 5% CO_2 in air .

@ Effector-to-target cell ratio.

Table 3.8 The effect of temperature on the induction of lymphocyte-mediated cytotoxicity against tumours by succinyl Con A

Tumour target	Incubation temperature*	E:T@	% Specific ^{51}Cr release	
			No sCon A	With sCon A
YAC-1	37°C	50:1	15.1±1.2	47.7±1.2
		100:1	23.7±0.5	62.1±1.8
	22°C	50:1	1.1±0.6	0.4±0.5
		100:1	2.2±0.5	2.6±1.1
	4°C	50:1	1.3±0.5	1.4±1.0
		100:1	0.9±0.9	1.4±0.1
MBL-2	37°C	50:1	4.4±0.9	50.0±0.4
		100:1	3.4±2.3	50.1±1.1
	22°C	50:1	0.8±0.3	1.4±0.6
		100:1	1.5±0.5	1.2±0.2
	4°C	50:1	2.0±0.4	0
		100:1	2.0±0.5	1.0±0.2

* Normal mouse splenocytes were incubated with ^{51}Cr labelled tumour target cells in the absence or presence of sCon A (6µg/ml) for 6 hrs (YAC-1 target) and 10 hrs (MBL-2 target) at the indicated temperatures in an air-tight box containing 5% CO_2 in air .

@ Effector-to-target cell ratio.

mouse splenocytes were incubated with ^{51}Cr-labelled tumour target cells in the absence or presence of lectin (Con A or sCon A). αmMan was also added at various concentrations and the mixtures were incubated for the indicated periods of time. As shown in Fig. 3.9 , sCon A-induced cytolysis of the two tumour targets was readily inhibited by the addition of a very low concentration of αmMan (1.6 mg/ml). In contrast, a much higher concentration of αmMan (12.5 mg/ml) was required to inhibit all LDCC induced by Con A. Therefore, these results indicated that the binding affinity of αmMan for Con A and sCon A may be quite different.

3.11 The requirement of divalent metal ions on lectin-induced lymphocyte-mediated cytotoxicity

It has been well established that calcium ions play a very important role on the antigen-specific T cell-mediated cytotoxicity (Plaut *et al.*, 1976). It is of interest to know whether they are also involved in LDCC. EGTA is a very commonly used Ca^{++} chelator which can be used to deplete extracellular Ca^{++} in the culture medium. In the first set of experiments, normal mouse splenocytes were incubated with ^{51}Cr-labelled tumour target cells in the absence or presence of Con A (6 μg/ml). EGTA at various concentrations was also added and the mixtures were incubated for the indicated periods of time. As shown in Fig. 3.10, it can be seen that EGTA exhibited a dose-dependent inhibitory effect on the Con A-dependent lymphocyte-mediated cytolysis of the two tumour targets. Very little inhibitory effect was seen at an EGTA concentration of 0.4 mM, however, a

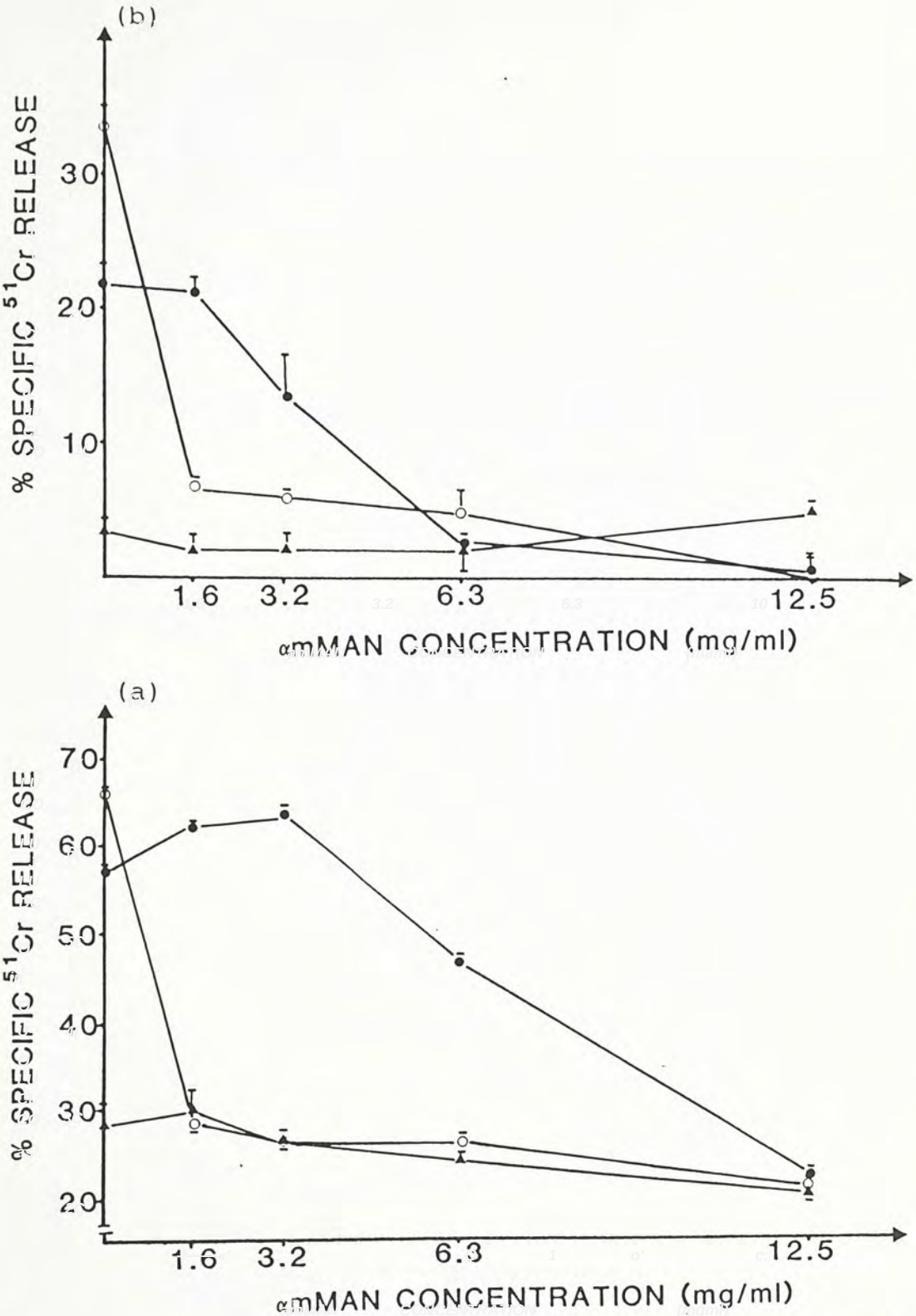

Figure 3.9 Dose-dependency of α-methyl-D-mannoside on inhibition of lymphocyte-mediated cytotoxicity induced by Con A and succinyl Con A. Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1 cells (a) and MBL-2 cells (b) in the presence of Con A (6 ug/ml) (●-●), sCon A (6 μg/ml) (O-O) or in the absence of the two lectins (▲-▲). αmMan was added at the beginning of the culture at various concentrations, the mixtures were incubated for 6 hrs (a) or 10 hrs (b) and the E:T ratio was 100:1.

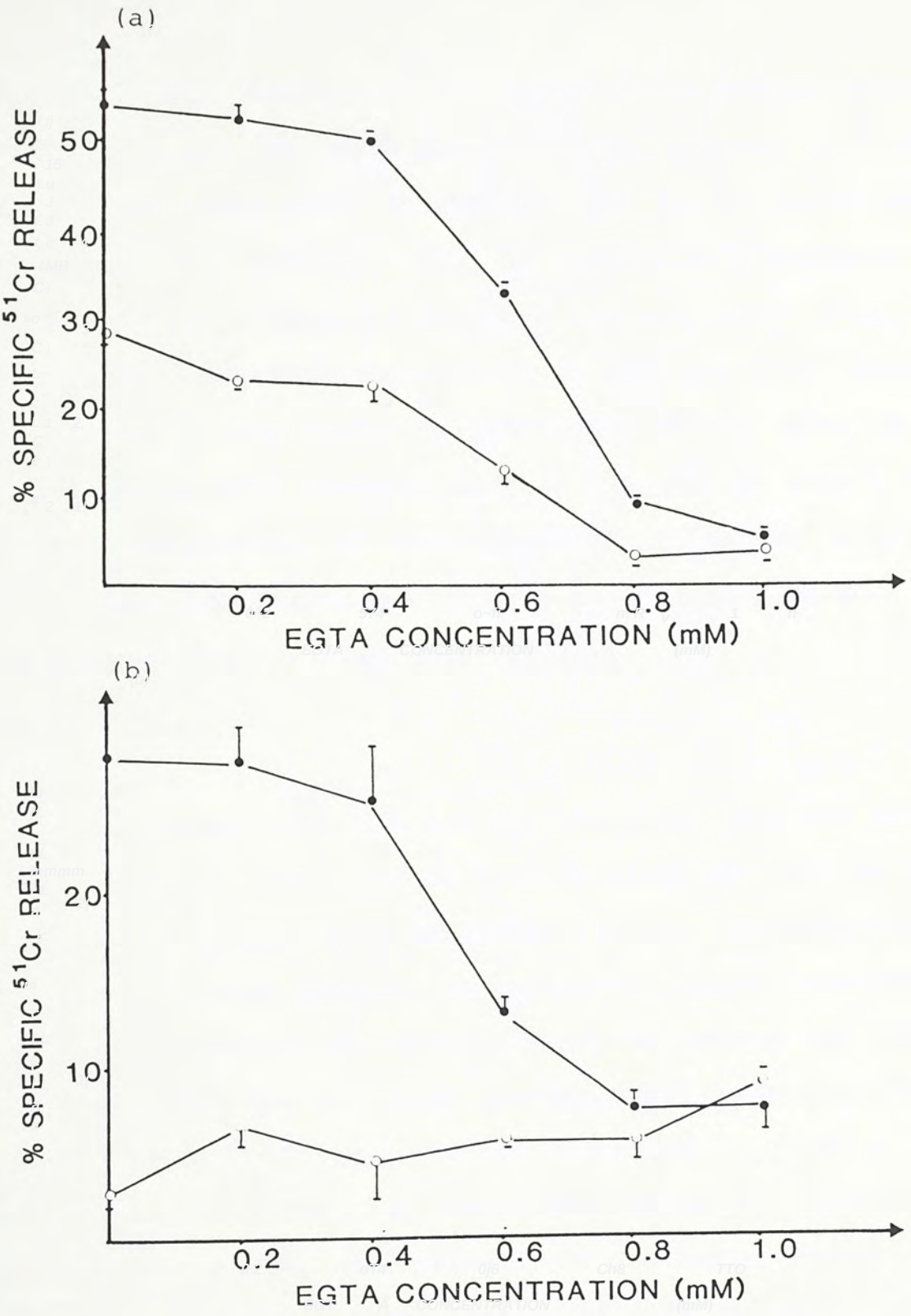

Figure 3.10 The effect of depletion of Ca^{++} by EGTA on Con A-induced cytolysis. Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) tumour target cells at an E:T ratio of 100:1 in the absence (○-○) or presence (●-●) of Con A (6 µg/ml). EGTA at various concentrations was also added and the mixtures were incubated for 6 hrs(a) and 10 hrs (b).

very significant decrease in the lysis of the tumour targets was observed at 0.6 mM EGTA concentration whereas 0.8 mM EGTA virtually abolished (>90% inhibition) the Con A-induced cytolysis of the two tumour targets. Similar results were obtained for the sCon A-induced lymphocyte-mediated cytotoxicity (Fig. 3.11) except that the inhibitory effect of EGTA was less drastic on YAC-1 target (only about 80% inhibition of the sCon A-induced cytolysis at an EGTA concentration of 0.8 mM). It should be noted that the endogenous NK activity in YAC-1 target also showed similar dose-inhibition curves with the addition of EGTA to the assay system (Fig. 3.10(a) and 3.11 (a)).

It is known that EGTA can chelate other divalent metal ions as well as Ca^{++}. In order to further study the role of Ca^{++} and other divalent metal ions on lectin-induced lymphocyte-mediated cytolysis of tumour targets, in the second set of experiments, Ca^{++} or Mg^{++} were examined for their ability to reverse the inhibitory effect on LDCC due to metal ion depletion resulting from the addition of EGTA (0.8mM). It was found that 0.13 mM Ca^{++} could significantly reverse the inhibitory effect of EGTA on LDCC against MBL-2 target and virtually no inhibitory effect of EGTA was observed at a Ca^{++} concentration of 0.25 mM (Fig. 3.12 (a)). In contrast, Mg^{++} were found to be ineffective in reversing the inhibitory effect of EGTA on LDCC against MBL-2 target even when present at a concentration as high as 1 mM (Fig 3.12 (b)). Similar results were obtained when sCon A was used as the lectin

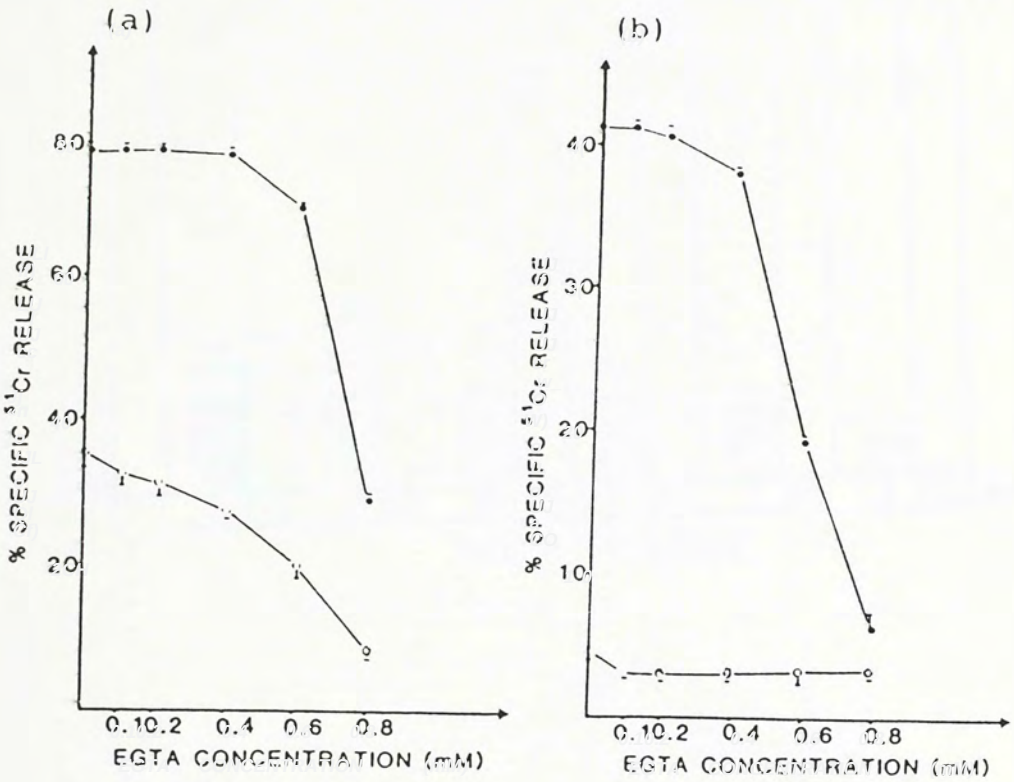

Figure 3.11 Effect of depletion of Ca^{++} by EGTA on succinyl Con A-induced cytolysis. Normal mouse splenocytes were incubated with ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) tumour target cells at an E:T ratio of 100:1 in the absence (O-O) or presence (●-●) of sCon A (6 µg/ml). EGTA at various concentrations was also added and the mixtures were incubated for 6 hrs (a) and 10hrs(b).

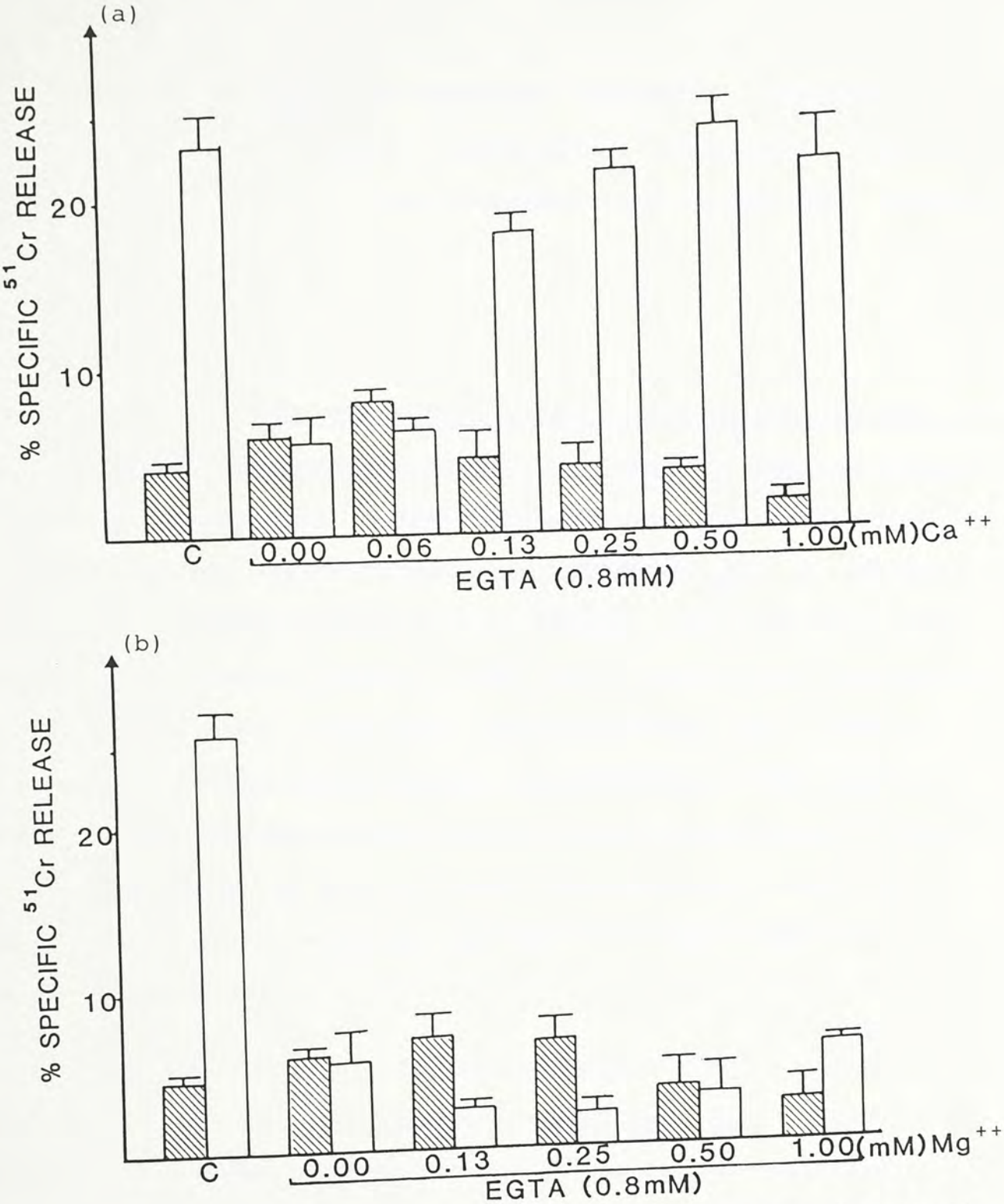

Figure 3.12 The effect of divalent metal ions on Con A-induced lymphocyte-mediated cytotoxicity. Normal mouse splenoyctes were incubated with ^{51}Cr-labelled MBL-2 tumour target cells in the absence (hatched bar) or presence (open bar) of Con A (6 µg/ml) at an E:T ratio of 100:1. With the exception of the control group (C), EGTA was added to the mixtures at a final concentration of 0.8 mM. Ca^{++} (a) and Mg^{++} (b) at various concentrations were then added (except the control group) and the specific ^{51}Cr-release was determined in a 10 hr assay.

for induction of lymphocyte-mediated cytotoxicity against MBL-2 tumour target (Fig. 3.13). These data , when taken together, indicate that Ca^{++} may play a crucial role on the LDCC against tumours.

3.12 The inhibitory effect of various pharmacologic agents on lectin-induced lymphocyte-mediated cytotoxicity against tumours

As a preliminary attempt to elucidate the mechanisms by which lectins can induce normal splenocytes to lyse a syngeneic tumour target, several pharmacologic agents were tested for their ability to inhibit LDCC. The full names and biological activities of these drugs were listed in Table 3.9. The drugs at their pre-determined optimal concentrations for inhibiting lymphocyte proliferation were used in the present study. It was found that the drugs used at these concentrations were neither toxic to normal splenocytes nor to the tumour target within 12 hrs (data not shown).

In order to study the effect of various pharmacologic agents on LDCC, normal C57 BL/6J mouse splenocytes were incubated with syngeneic ^{51}Cr-labelled MBL-2 cells in the absence or presence of lectin (Con A or sCon A, 6 μg/ml). Various drugs at the indicated concentrations were also added and the mixtures were incubated for 10 hrs.

As shown in Fig. 3.14 and 3.15, EGTA and αmMan were very potent inhibitors of LDCC against MBL-2 target whereas both H7

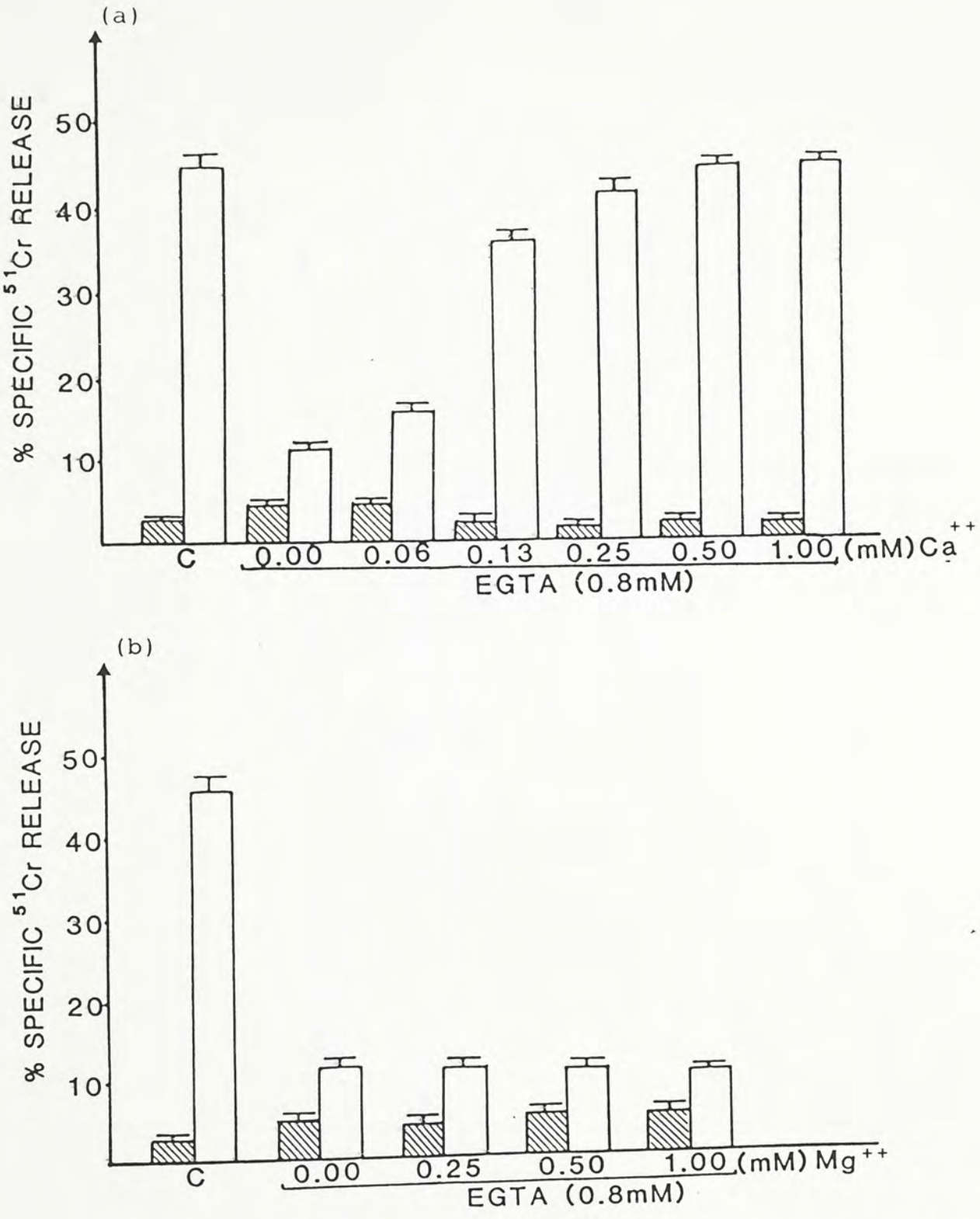

Figure 3.13 The effect of divalent metal ions on succinyl Con A-induced lymphocyte-mediated cytotoxicity. Normal mouse splenocytes were incubated with ^{51}Cr-labelled MBL-2 tumour target cells in the absence (hatched bar) or presence (open bar) of sCon A (6 µg/ml) at an E:T ratio of 100:1. With the exception of the control group (C), EGTA was added to the mixtures at a final concentration of 0.8 mM. Ca^{++} (a) and Mg^{++} (b) at various concentrations were then added (except the control group) and the specific ^{51}Cr-release was determined in a 10 hr assay.

Table 3.9 Pharmacologic agents used in the present study

Drugs	Full Name	Function	Optimal Concentration
H7	1-(5-isoquinolinyl sulfonyl)-2-methylpiperazine	Protein Kinase C Inhibitor	100uM
W7	N-(6-aminohexyl)-(5-chloro-1-naphthalen-sulfonamide	Calmodulin Antagonist	10uM
NEO	Neomycin	IP Turnover Inhibitor	10mM
EGTA	Ethylene glycol bis(beta-amino-ethyl ether)-N,N,N',N'-tetra-acetic acid	Ca^{++} Chelator	0.8mM
αmMan	α-Methyl-D-mannoside	Con A Inhibitor	12.5mg/ml

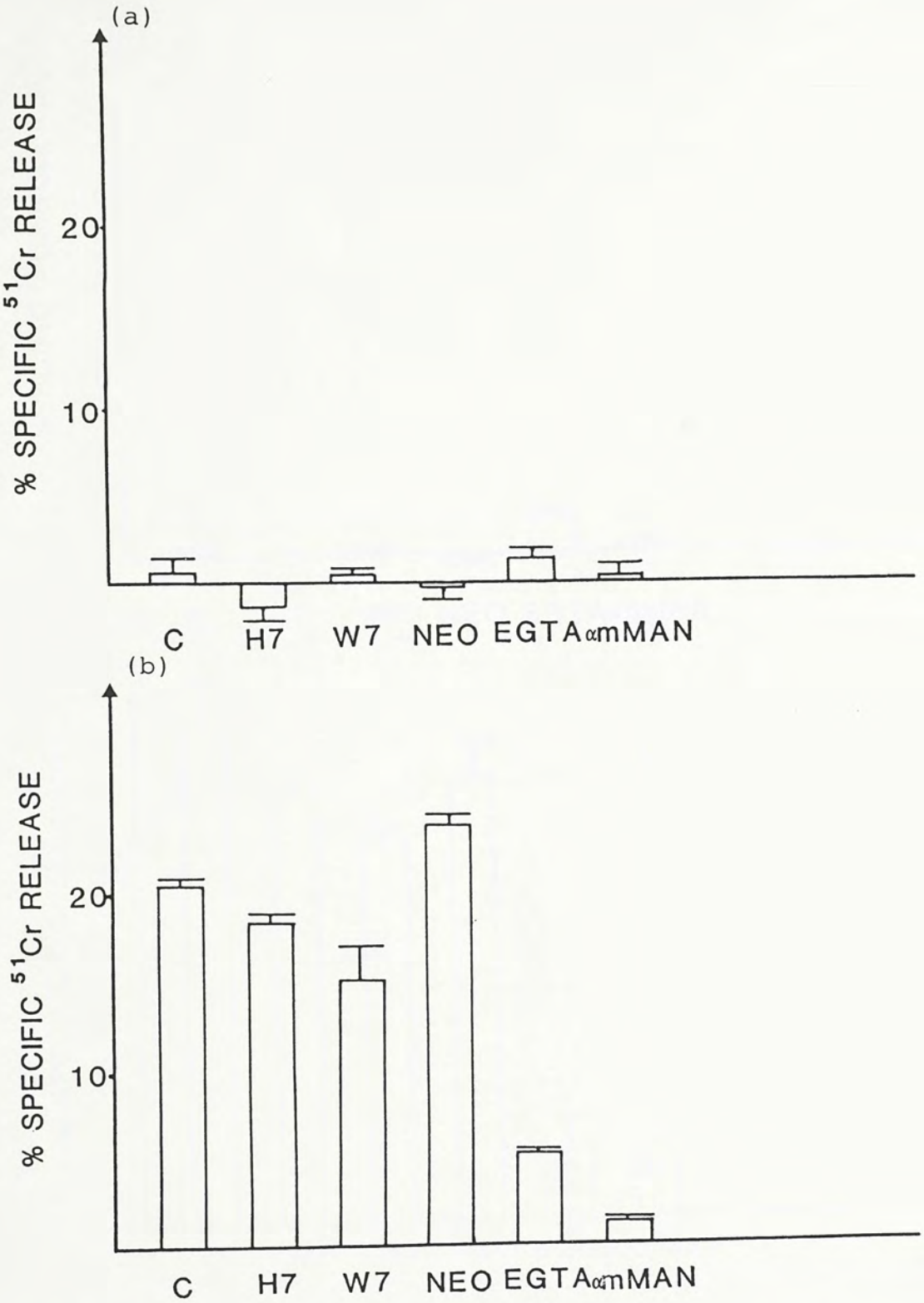

Figure 3.14 The inhibitory effect of various pharmacologic agents on Con A-induced lymphocyte-mediated cytotoxicity against syngeneic tumour. Normal C57 BL/6J mouse splenocytes were incubated with ^{51}Cr-labelled MBL-2 target cells at an E:T ratio of 100:1 in the absence (a) or presence (b) of Con A (6 µg/ml). Various pharmacologic agents at the indicated concentrations were also added and the mixtures were incubated for 10 hrs.

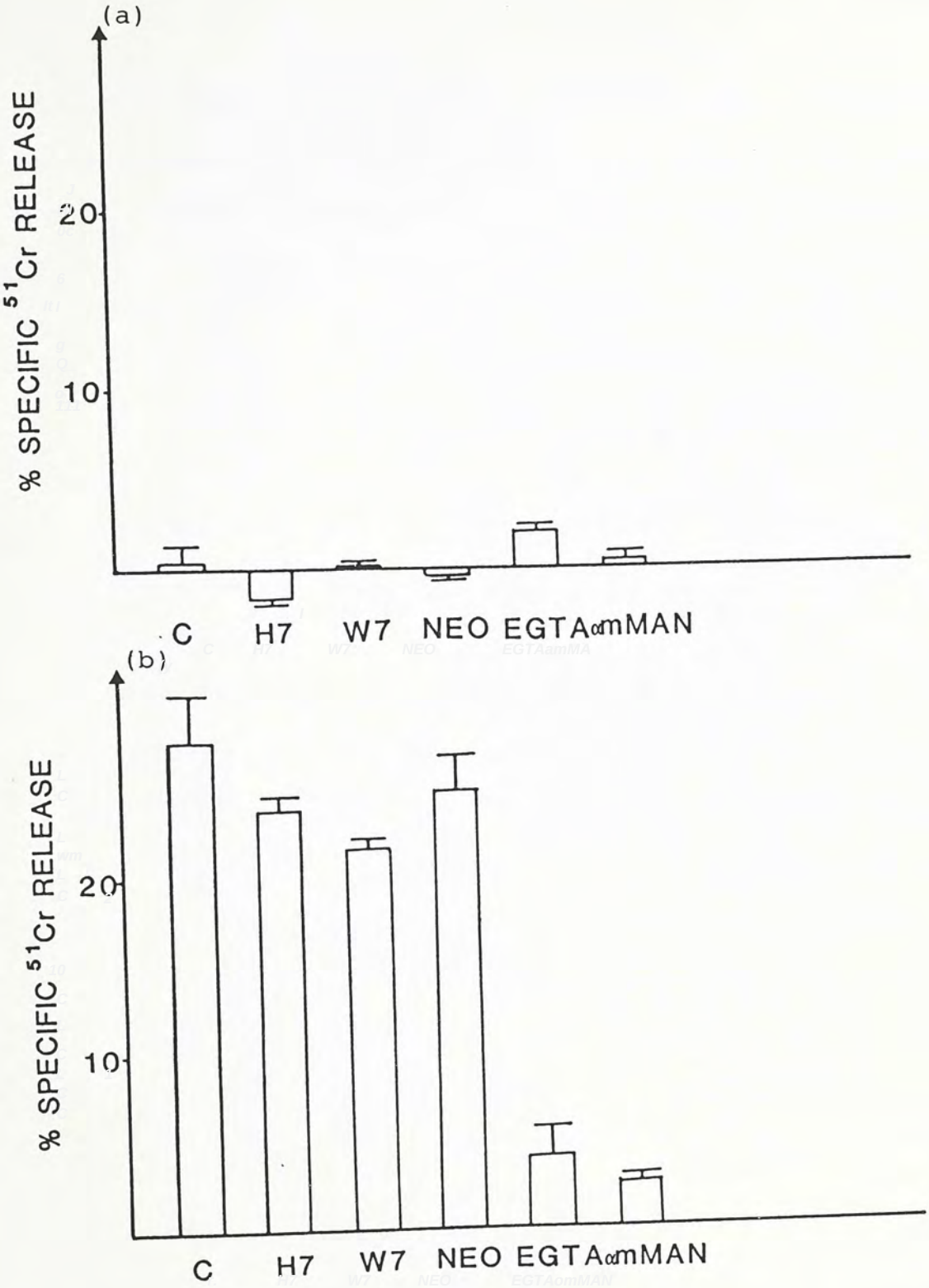

Figure 3.15 The inhibitory effect of various pharmacologic agents on succinyl Con A-induced lymphocyte-mediated cytotoxicity against syngeneic tumour. Normal C57 BL/6J mouse splenocytes were incubated with ^{51}Cr-labelled MBL-2 cells at an E:T ratio of 100:1 in the absence (a) or presence (b) of sCon A (6 µg/ml). Various pharmacologic agents at the indicated concentrations were also added and the mixtures were incubated for 10 hrs.

and W7 showed only a slight, though significant, inhibition of the lectin-induced cytolysis of this tumour target. In contrast, NEO seemed to have no inhibitory effect on LDCC against MBL-2 cells.

DISCUSSION

A number of earlier reports (Moller, 1965; Rubens and Henney 1977; Thierry et al.,1977; Tartof and Fitch, 1978; Waterfield et al., 1981; Eugene et al. 1982)had shown that only alloimmune lymphocytes or Con A or PHA-activated lymphocytes could kill syngeneic and allogeneic tumour cells in the presence of lectins. Significant cytolysis, however, was not detected when normal splenocytes were used as the effector cells. In contrast, the present investigation has demonstrated that mitogenic lectins such as PHA, Con A and succinyl Con A are quite capable of inducing normal mouse splenocytes to lyse a variety of syngeneic and allogeneic tumour targets. These results are in line with a recent report which showed that freshly isolated human peripheral blood mononuclear cells (PBMC) were capable of mediating strong cytotoxicity against adherent HEp-2 cells in the presence of Con A (Perl et al., 1984). Although the reason for the apparent discrepancy of the present findings with that of others is unclear. This may be attributed to the fact that most of the earlier investigators had used a very short incubation time (usually 4 hrs or less) and/or a very high dose of Con A (usually 20 µg/ml) for their assays. In contrast, the present results showed that the optimal concentration of Con A for the induction of normal splenocyte cytotoxicity was about 3-6 µg/ml. In addition, maximum lysis occurred at 6-12 hrs after incubation of effector and target cells, depending on the type of target cells being used. Moreover, the tumour targets commonly used by others were mainly EL-4 and P815 cells, which were found to be

relatively poor targets in my experimental system.

In order to study the induction of normal splenocyte cytotoxicity by lectins, normal splenocytes were incubated with ^{51}Cr-labelled tumour target cells in the presence of various concentrations of lectins. It was found that both native lectins (Con A and PHA) and lectin derivative such as succinyl Con A (sCon A) could induce significant normal splenocyte cytotoxicity against tumour targets in a dose-dependent manner. It can be seen that both Con A and sCon A were much more potent than PHA for the induction of LDCC. Moreover, different tumour targets showed different susceptibility to lectin-induced cytolysis. The present results indicated that the syngeneic, NK-insensitive MBL-2 cells and the allogeneic, NK-sensitive YAC-1 cells were efficiently killed by normal C57 BL/6J mouse splenocytes in the presence of lectins when compared to that of the WEHI-3 and P815 cells which were of intermediate sensitivity to lysis. EL-4 cells, on the other hand, were found to be a poor target for LDCC since very low levels of cytotoxicity were induced by the lectins. The difference in susceptibility of these tumour targets may well be explained by the fact that different tumour cells have different surface components such as glycoproteins and glycolipids so their interactions with lectins may be quite different.

I have compared the induction of LDCC by Con A to its succinylated derivative. Unlike that of Con A, the sCon A-induced cytotoxicity against MBL-2 and YAC-1 tumour targets was not

diminished at higher concentrations of the lectin. Similarly, Hadden et al. (1976) had shown that sCon A did not exhibit diminished mitogenic action at higher concentrations as compared to that of Con A. Although Con A and sCon A are both mitogenic for murine and human lymphocytes (Gunther et al., 1973, Hadden et al., 1976), it has been reported that sCon A differs from native Con A in that it is 10 fold less agglutinating for lymphocytes, it does not induce patch and cap formation and does not inhibit anti-Ig induced capping (Edelman, 1974). Why the dose-response curves of LDCC induced by Con A and sCon A are different is not entirely clear. Perhaps this may be caused by the conversion of Con A from the tetrameric to the dimeric form as a result of succinylation (Gunther et al., 1973). It is conceivable that the reduced induction of LDCC by Con A at higher concentrations may be due to the extensive crosslinking of cell surface receptors since Con A is tetrameric at pH above 5.6. Succinyl Con A, on the other hand, remains dimeric at pH above 5.6 and as a result of the reduced valence of sCon A, extensive crosslinking of receptors may not occur at higher lectin concentrations and thus a diminished induction of LDCC was not observed. Alternatively, the difference observed with sCon A and Con A may also be due to the alteration in the charge of Con A induced by succinylation. Moreover, unlike Con A, it was found that sCon A did not increase the level of cyclic AMP at higher concentrations (Hadden et al., 1976). Since cyclic AMP is an important second messenger in many pathways of cellular activation, increasing concentrations of cyclic AMP may also influence the induction of LDCC by Con A.

This possibility is supported by a recent report which showed that pretreatment of CTL with cholera toxin or other agents that raised the intracellular level of cAMP in CTL had resulted in a partial inhibition of the T cell receptor (TcR)-triggered "lethal hit" delivery to the target cell, and almost completely blocked the TcR-triggered exocytosis of granules from CTL (Takayama et al., 1988). However, whether the reduced ability of Con A at higher concentrations to induce LDCC is due to the increased level of intracellular cAMP in this system remains to be determined.

In order to characterize the nature of effector cells mediating LDCC, mouse splenocytes were either stimulated in vivo with poly I:C or inactivated in vitro with mitomycin C. It was found that both poly I:C-stimulated splenocytes and MMC-treated splenocytes were equally effective in mediating cytotoxicity against tumours in the presence of lectins when compared to that of the normal, untreated splenocytes. These results indicate that neither prior activation of effector cells in vivo nor proliferation of effector cells in vitro is a prerequisite for induction of LDCC. On the other hand, whether de novo RNA and protein synthesis may play a role on the induction of LDCC has yet to be determined.

To determine the cell type(s) that is responsible for mediating LDCC, normal mouse splenocytes were either depleted of T cells by treatment with anti-Thy 1.2 antibody and complement or enriched for T cells by passage through nylon wool column. The

data clearly showed that the lectin-induced cytolysis of tumour targets was completely abrogated by anti-Thy 1.2 antibody and complement treatment whereas enrichment of T cells had resulted in increased levels of lectin-induced cytotoxicity against tumours. These results, when taken togehter, strongly suggest that the lectin-induced cytotoxicity is mediated by T cells. Such a finding is consistent with many others who showed that the major effector cells in LDCC are T lymphocytes (Bevan and Cohn, 1975; Rubens and Henney, 1977; Schuber and Lucas, 1981; Bonavida et al., 1983; Perl et al., 1986). However, it has been reported that monocytes and polymorphonuclear cells can also mediate LDCC and that the choice of lectins and targets is of primary importance in connection with which effector cells are involved in a given LDCC system (Jondal and Targan, 1978; Yue et al., 1981; Yamazaki et al., 1983).

It is unclear how lectins can induce normal splenocytes to kill a syngeneic or allgeneic tumour. Some investigators suggested that the lectin served merely as a bridge, bringing an inherently lytic T cell into close approximation with the target cell (Forman and Moller, 1973; Bevan and Cohn, 1975). Other studies, however, demonstrated that some agglutinating agents, while bringing the effector and target cells in close contact, were not able to mediate LDCC (Bonavida and Bradley, 1976; Green et al., 1978). Therefore, it is of great interest to examine whether lectin pretreatment of the effector and/or target cells would support cytolysis. The present results clearly showed that

pretreatment of either effector or target cells with succinyl Con A failed to induce significant LDCC against the two tumour targets. This would seem to indicate that the mere 'activation' or `modification' of either effector or target cells alone by sCon A is insufficient to induce cytolysis and that the continuous presence of sCon A or the bridging of the effector to the target is essential for cytolysis to occur. On the contrary, pretreatment of either effector or target cells with Con A could induce significant LDCC against the two tumour targets. There are at least two likely explanations for this observation. Firstly, Con A activation or modification of either the effector or target cells alone is sufficient to mediate LDCC. Alternatively, since Con A is polyvalent, it could still interact with both effector and target cells such that bridging would have occurred even if both pretreatment and washing were performed. Of course the possibility exists that Con A may serve a dual role in this process : activation of effector or target and bridging the effector to its target. Clearly, more experiments are required in order to distinguish these possibilities.

As initial attempts to elucidate the mechanism of lectin-induced lymphocyte-mediated cytotoxicity against tumours in the present system, I have studied the temperature-dependence and calcium ions requirement in the cytolysis induced by the lectins. Moreover, the effect of various pharmacologic agents on the induction of LDCC was also examined. It has been well documented that the antigen-specific T cell-mediated cytotoxicity is a temperature-dependent process (Berke, 1980). Similarly, the

present data showed that the lectin-induced cytolysis only occurred at 37 °C while cooling the incubation mixture to 4 °C or by maintaining the effector and target cells at room temperature failed to induce any significant lysis. It is conceivable that temperature may affect LDCC in at least three ways : 1. the activation of the cellular response that may be required for killing to occur; 2. the binding of lectins to the surface of the effector and target cells; and 3. the target cell disintegration (release of ^{51}Cr) process.

It has been well established that Ca^{++} play an important role in many cellular activation processes. The addition of the calcium-chelating agent EGTA to the assay system is known to inhibit the antigen-specific cytotoxicity (Plaut et al., 1976). In this investigation, it was found that EGTA at 0.8 mM concentration could inhibit nearly all the lectin-induced cytotoxicity against tumours, indicating that Ca^{++} may play a crucial role in this process. This is further supported by the fact that the addition of Ca^{++} but not Mg^{++} was effective in reversing the inhibitory effect of EGTA on LDCC. This raised the question as what role(s) Ca^{++} play in LDCC against tumours. Obviously, there are several possibilities. Firstly, Ca^{++} may affect cell-cell interaction. Secondly, Ca^{++} may be involved in lectin binding to effector and target cells. Thirdly, Ca^{++} may be involved in intracellular signalling since there is an increased calcium influx into the effector cells during the early events of mitogen triggering (Whitney et al., 1972). Fourthly,

Ca^{++} may be directly involved in the early stages of Ca^{++} - dependent lytic processes. Therefore the exact role(s) that Ca^{++} play in LDCC has yet to be established.

Thus it can be seen that the cytolysis induced by lectins has requirements for temperature and calcium ions similar to that of the antigen-specific T cell-mediated lysis. As temperature and Ca^{++} have been shown to influence the reorientation of the perinuclear Golgi apparatus and the microtubule-organizing center (Kupfer et al., 1985), perhaps this reorientation process may also be important in the lectin-induced cytolysis of tumours.

To determine whether the binding of the lectins (Con A, sCon A) to the effector or target cell surface is a prerequisite for triggering cytolysis, α-methyl-D-mannoside, an effective inhibitor of Con A, was used in the present study. It is known that αmMan can rapidly bind to Con A and remove it from the cell surface (Powell and Leon, 1970). It was found that only a high concentration of αmMan (12.5 mg/ml) was effective in inhibiting the LDCC induced by Con A whereas a much lower concentration (0.63 mg/ml) of αmMan was sufficient to inhibit all LDCC induced by sCon A. These results would seem to suggest that the binding affinity of sCon A for effector and target cells was much weaker than that of Con A and hence αmMan could bind to sCon A and readily displaced it from the cell surface much more efficiently as compared to that of Con A.

In order to have a better insight into the mechanism of

LDCC, several pharmacologic agents were used to inhibit or interfere with the essential processes that are involved in the intracellular signalling events during cell activation. H7 is an inhibitor of protein kinase C which is a main enzyme involved in cell activation whereas the W7 is a calmodulin antagonist. These two drugs at their optimal concentrations (100 uM and 10 uM respectively) could significantly inhibit normal splenocyte proliferation whereas the same concentrations of drugs only slightly inhibited the LDCC induced by the two lectins. Similarly, neomycin is an inositol phosphate (IP) turnover inhibitor which could also inhibit cell proliferation when present at a concentration of 10 mM. However, the same concentration of this drug seemed to exert no inhibitory effect on LDCC against the MBL-2 tumour. A likely explanation for these findings is that the induction of LDCC by lectins may involve several pathways so inhibition of one pathway may not affect greatly the results. This possibility is currently being investigated in our laboratory.

In conclusion, mitogenic lectins such as Con A and succinyl Con A can induce significant splenocyte cytotoxicity against various syngeneic and allogeneic tumours. The lectin-induced effector cells are T cells. Cytolysis is both calcium ions and temperature-dependent. The question remains open as to what the lectin is providing in the cytotoxic assay. However, cytolysis is abolished by the addition of α-methyl-D-mannoside to the assay system indicating that the binding of the lectins to the effector and/or target cell surface may be critical for lysis to occur.

CHAPTER FOUR : INDUCTION OF NONSPECIFIC LYMPHOCYTE-MEDIATED CYTOTOXICITY BY PHORBOL ESTER AND/OR CALCIUM IONOPHORE

CONTENTS

INTRODUCTION

It has been well documented that cell-mediated cytotoxicity depends on transduction signals to transform the initial effector-target cell contact into a fully lytic event (Berke, 1983). For antigen-specific cytotoxic T lymphocytes (CTL), this depends on the capacity of the T cell receptor for antigen (TcR) on the effector cell surface to recognize and bind to the relevant antigen on the target cell surface so as to transmit this recognitive event to the interior of the CTL and then activate it (Isakov et al.,1987). Other structures such as Lyt-2 or L3T4 molecular complexes and LFA-1 molecule may also contribute to the overall avidity of interaction between effector and target cells in the murine system but the mechanism by which this occurs is not clear (Davignon et al., 1981; MacDonald et al., 1982). Recent studies suggest that a signal transduction pathway involving inositol phospholipid hydrolysis is important in T cell activation and function. In this pathway, receptor occupation by a ligand stimulates a phospholipase C to hydrolyze membrane bound phosphatidylinositol 4,5-bisphosphate into two products : the diacylglycerol(DG) and inositol 1,4,5-trisphosphate(IP_3). DG activates protein kinase C (PKC) by increasing its affinity for Ca^{++} while IP_3 mobilizes Ca^{++} from the endoplasmic reticulum, thereby increasing the free intracellular Ca^{++} concentration. The activation of PKC and elevation of cytoplasmic free Ca^{++} may both play important roles in the early events of signal transduction in T lymphocytes (Berridge and Irvine, 1984; Nishizuka, 1986).

Pharmacological agents such as phorbol esters and calcium ionophores are particularly useful for studying the molecular events of T cell activation as they apparently act directly on intracellular targets rather than through antigen-specific receptors on the T cell surface. Phorbol esters such as PMA and TPA have been demonstrated to provide an antigen-like activation signal to T cells and this is presumably mediated through PKC activation (Nishizuka, 1984; 1986). On the other hand, calcium ionophores such as A23187 and ionomycin have been used to induce artificial increases in cytoplasmic Ca^{++} levels in T lymphocytes (Maino et al., 1974; Mastro and Smith, 1983).

It has been reported that with murine CTL clones, calcium ionophore and phorbol ester can substitute for antigen in the induction of killer cells from primed precursors (Albert et al., 1985; Isakov and Altman, 1985). Lancki et al. (1987), on the other hand, have shown that cloned murine CTL can also be triggered by phorbol ester together with a calcium ionophore to lyse syngeneic or third party target cells efficiently. Interestingly enough, Ju et al. (1987) demonstrated that PMA and A23187 syngergistically activated murine $L3T4^{+}$ clones to express potent nonspecific cytolytic activity. Moreover, Mikael et al. (1987) have shown that TPA plus A23187 can directly induce human lymphocytes to kill certain tumour targets nonselectively. In view of these observations, it is of particular interest to examine whether normal mouse lymphocytes can be similarly triggered by PMA and/or A23187 to lyse various syngeneic and allogeneic tumour cell lines. In the present study, it was found

that PMA but not A23187 alone was able to trigger normal splenocytes to mediate cytolysis against various tumour targets. In addition, the PMA-induced cytotoxicity was found to be significantly enhanced with the simultaneous presence of A23187.

RESULTS

4.1 Dose response of drug-induced lymphocyte-mediated cytotoxicity against tumours

In order to determine whether phorbol ester (PMA) and/or calcium ionophore (A23187) can induce normal mouse splenocyte to lyse tumour targets nonspecifically *in vitro*, normal splenocytes from C57 BL/6J mice ($H\text{-}2^b$) were preincubated with different concentrations of two drugs either alone or in various combinations for 1 hr at 37°C. The ^{51}Cr-labelled allogeneic YAC-1 cells ($H\text{-}2^a$) or syngeneic MBL-2 cells ($H\text{-}2^b$) were then added and the mixtures were further incubated at 37°C for 6 hrs and 10 hrs respectively. The specific lysis of the these two tumour targets was then determined.

Data from Figure 4.1 showed that PMA at a concentration of 6.3 ng/ml significantly enhanced the natural splenocyte-mediated cytotoxicity against YAC-1 target. Such an enhancement of splenocyte killing on YAC-1 cells, however, was less marked at higher concentrations of PMA. In contrast, at lower concentrations (e.g. 1.6 ng/ml) of PMA, a slight suppression of the endogenous natural killing on YAC-1 target was observed. On the other hand, PMA alone at high concentrations (25-100ng/ml) induced significant, albeit low, splenocyte killing on MBL-2 target whereas at lower concentrations, direct induction of splenocyte-mediated cytotoxicity against MBL-2 cells was not

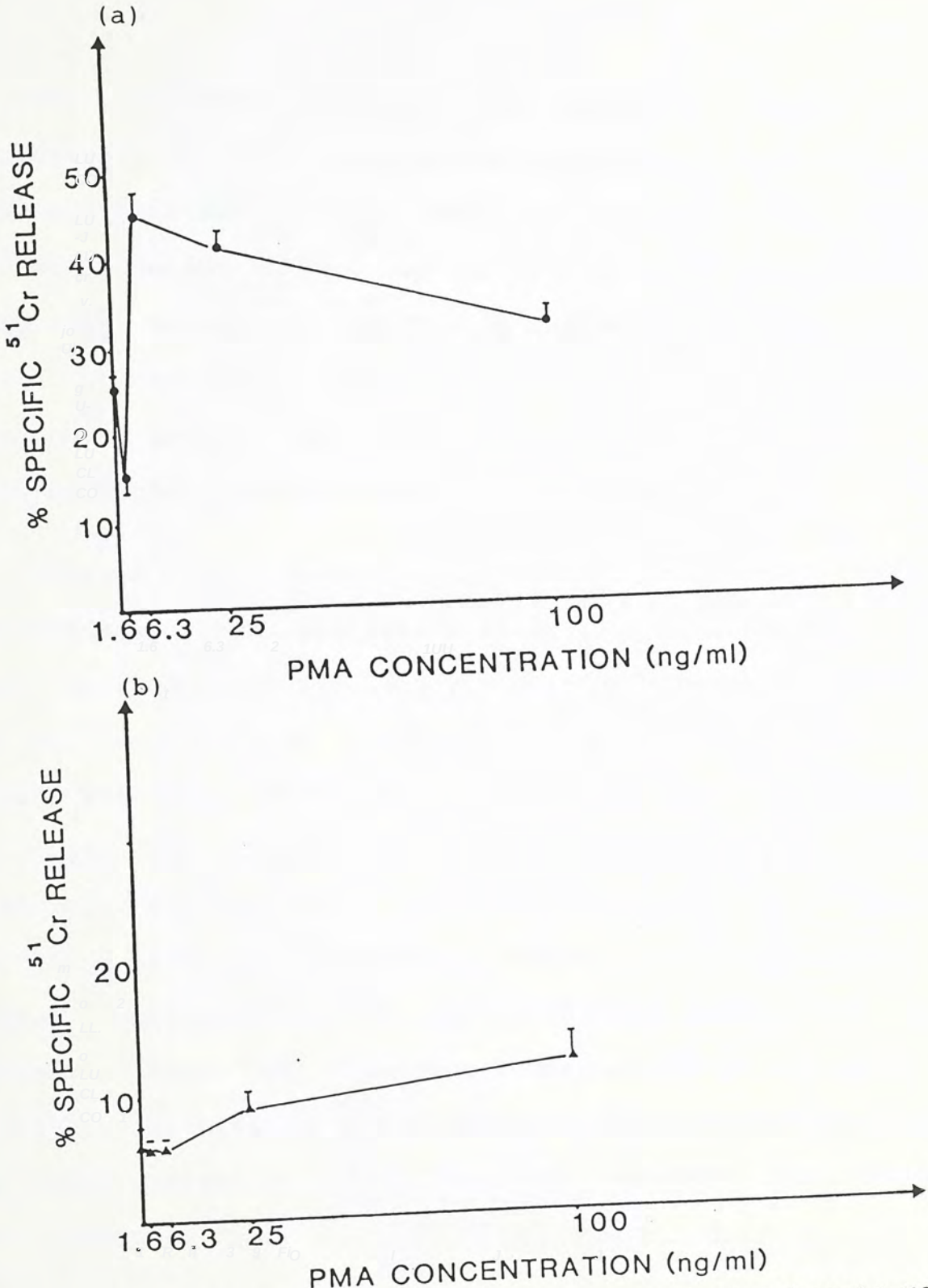

Figure 4.1 Dose response of PMA-induced lymphocyte-mediated cytotoxicity on YAC-1 and MBL-2 targets. Normal mouse splenocytes were preincubated with various concentrations of PMA for 1 hr at 37 °C before the addition of ^{51}Cr-labelled Yac-1 (a) and MBL-2 (b) tumour target cells. Cytolysis was determined in a 6 hr (a) or 10 hr (b) ^{51}Cr release assay and the effector-to-target cell ratio was 100:1.

seen.

The influence of calcium ionophore A23187 on the cytotoxicity of normal splenocytes towards the two tumour targets was investigated. Unlike PMA, it was found that A23187 at nontoxic concentrations (up to 2.5 μM) did not induce any detectable splenocyte killing on the MBL-2 tumour target (Fig. 4.2). In contrast , it exhibited an inhibitory effect on the endogenous natural killer activity of the splenocytes on YAC-1 cells at higher concentrations (1.25-2.5 μM).

Results in Figure 4.3 clearly indicated that the combinations of PMA and A23187 at various concentrations in most cases induced higher splenocyte cytotoxicity against the two tumours targets when compared to that induced by the same concentration of PMA alone. For YAC-1 target, a combination of 6.3 ng/ml PMA and 0.63 μM A23187 induced the highest level of splenocyte cytotoxicity whereas a combination of 25 ng/ml PMA and 2.5 μM A23187 was optimal for MBL-2 target. Therefore, these optimal combinations of PMA and A23187 were chosen for further studies. Since PMA alone at a concentration of 6.3 ng/ml could also induce significant lymphocyte-mediated cytotoxicity against the YAC-1 target so this was also included for subsequent investigations.

It should be noted that in the absence of effector cells, treatment of the two tumour targets with PMA and/or A23187 at all concentrations used in my experiments did not induce increased

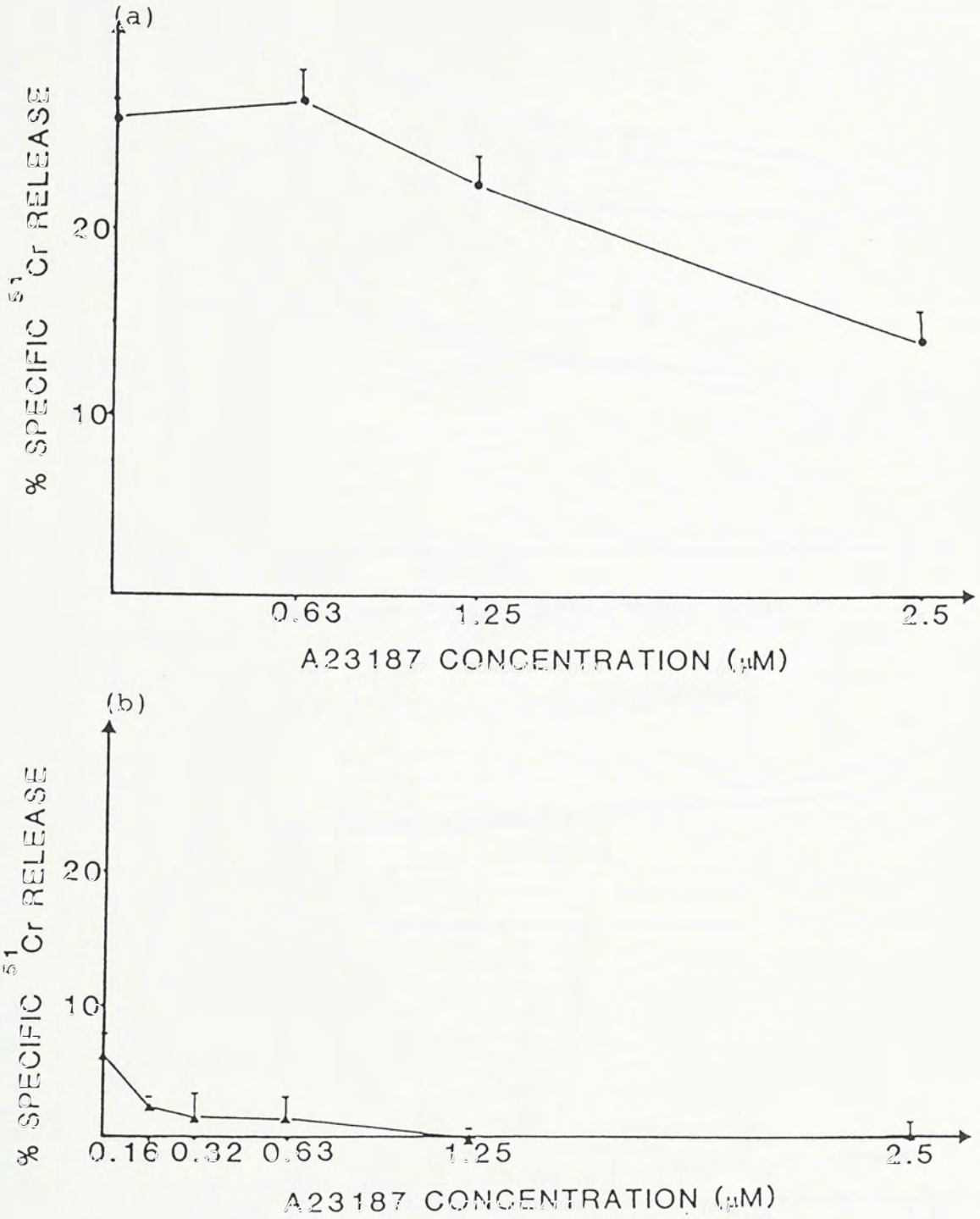

Figure 4.2 Dose response of A23187-induced lymphocyte-mediated cytotoxicity on YAC-1 and MBL-2 targets. Normal mouse splenocytes were preincubated with various concentrations of A23187 for 1 hr at 37°C before the addition of ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) tumour target cells. Cytolysis was determined in a 6 hr (a) or 10 hr (b) ^{51}Cr release assay and the effector-to-target cell ratio was 100:1.

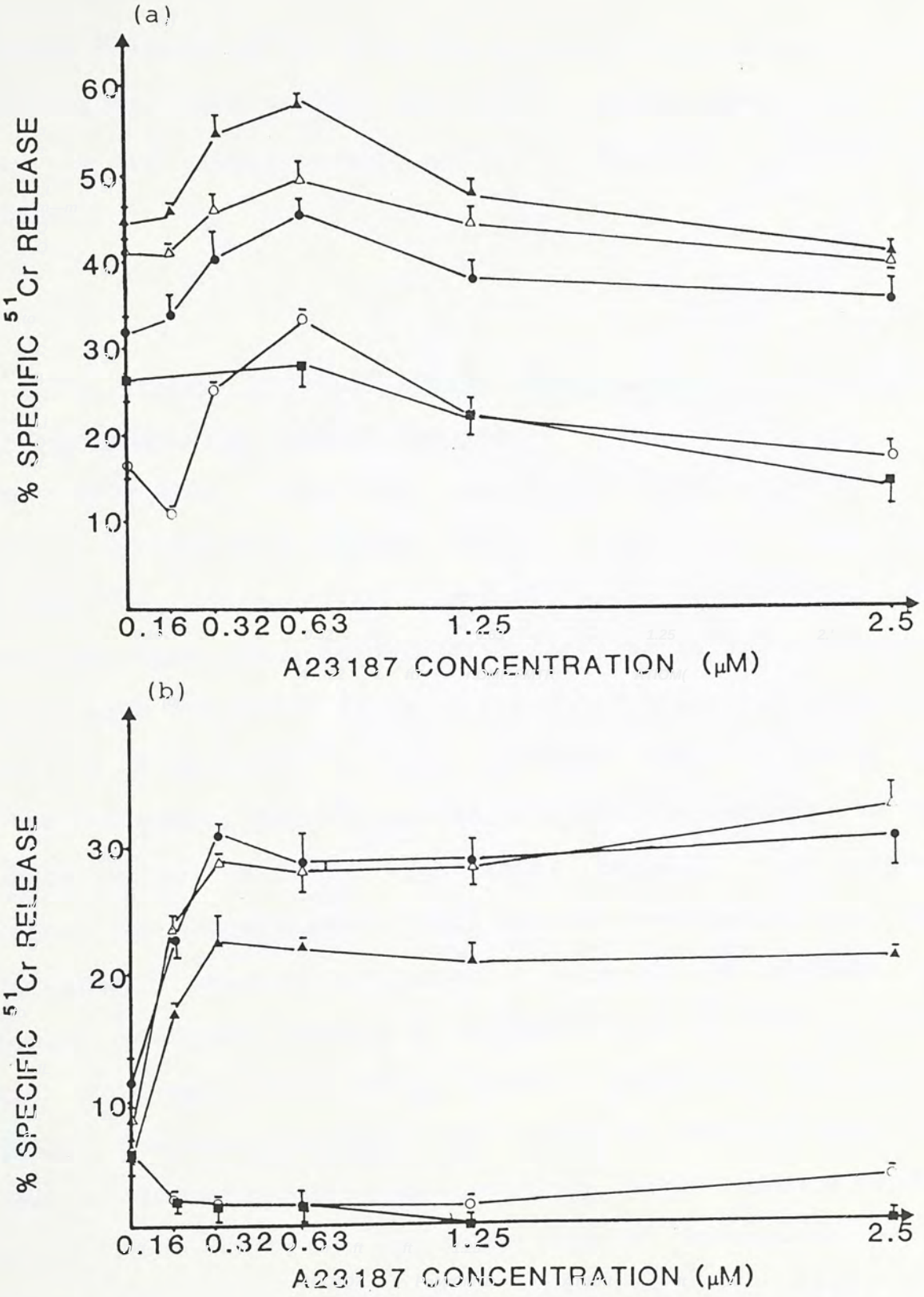

Figure 4.3 The effect of combinations of various concentrations of PMA and A23187 on the induction of lymphocyte-mediated cytotoxicity on YAC-1 and MBL-2 targets. Normal mouse splenocytes were preincubated with various concentrations of PMA (●-● 100ng/ml; △-△ 25ng/ml; ▲-▲ 6.3ng/ml; ○-○ 1.6ng/ml; ■-■ medium) and A23187 for 1 hr at 37°C before the addition of ^{51}Cr-labelled YAC-1 (a) and MBL-2 (b) tumour target cells. Cytolysis was determined in a 6 hr (a) or 10 hr (b) ^{51}Cr release assay and the effector-to-target cell ratio was 100:1.

spontaneous release of ^{51}Cr from the target cells, indicating that these two drugs, when present alone or in combinations, are not toxic to the two tumour targets under the prescribed experimental conditions.

4.2. Time course of PMA and/or A23187 -induced lymphocyte-mediated cytotoxicity against tumours

To determine the time course for the induction of splenocyte cytotoxicity against the two targets (YAC-1 and MBL-2 cells) by PMA and/or A23187, normal mouse splenocytes were preincubated with PMA and/or A23187 for 1 hr at 37°C and then incubated with ^{51}Cr-labelled YAC-1 and MBL-2 cells for various time periods (2-12 hrs). As shown in Figure 4.4, it can be seen that the PMA and/or A23187 induced cytolysis increased with time with both tumour targets. With YAC-1 target, the greatest difference in the percentage lysis between the control and PMA-treated groups reached a plateau at about 6 hrs whereas the percentage cytolysis induced by PMA+A23187 was maximal at about 10 hrs. However, with MBL-2 target, cytolysis induced by PMA+A23187 increased progressively with time and a plateau was not seen up to 12 hrs of incubation (Fig. 4.4). Since in many pioneering experiments it was found that an incubation period exceeding 10 hrs was often associated with high spontaneous lysis, therefore, a 6 hr ^{51}Cr-release assay was chosen for YAC-1 cells and a 10 hr ^{51}Cr-release assay was used for MBL-2 cells in subsequent experiments.

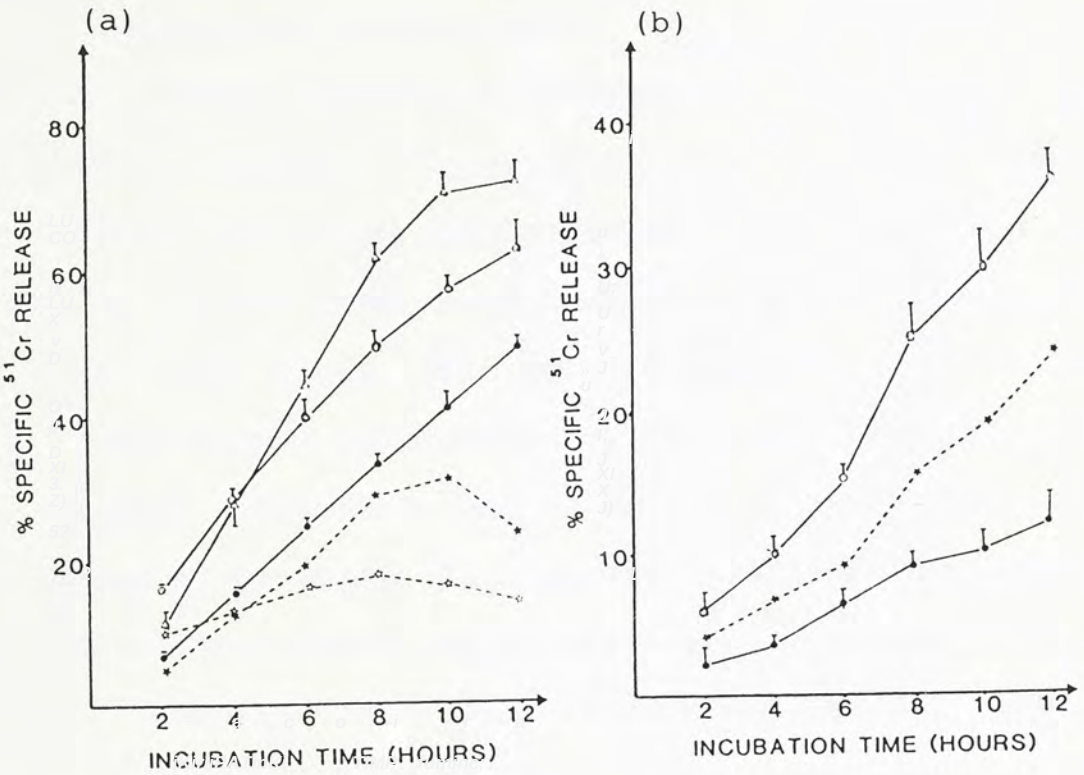

Figure 4.4 Time course of PMA and/or A23187-induced lymphocyte-mediated cytotoxicity against tumours. Normal mouse splenocytes were preincubated with PMA (6.3ng/ml) (○–○); PMA (6.3ng/ml)+ A23187 (0.63 μM) (△–△) or medium (●–●) for 1 hr at 37°C and then incubated with ^{51}Cr-labelled YAC-1 target (a). Similarly, normal mouse splenocytes were preincubated with PMA (25 ng/ml) + A23187 (2.5 μM) (○–○) or medium (●–●) for 1 hr at 37°C and then incubated with ^{51}Cr-labelled MBL-2 target (b). The effector-to-target ratio was 100:1. The percentage lysis was determined every 2 hrs for 12 hrs. The differences between the percentage lysis of tumour target in the absence and presence of PMA (☆–☆) or PMA+A23187 (★–★) are referred as the PMA- or PMA+A23187- induced cytolysis.

4.3 The induction of lymphocyte-mediated cytotoxicity against various tumours by PMA and/or A23187

From the previous experiments, it was found that PMA and/or A23187 could induce splenocyte cytotoxicity against the YAC-1 and MBL-2 tumour targets. It is of interest to know whether PMA and/or A23187 can also induce normal splenocytes to mediate cytotoxicity against other tumour targets. Results in Table 4.1 showed that PMA alone was able to trigger a low level of lymphocyte-mediated splenocyte cytotoxicity against various tumour targets including P815, EL-4 and WEHI-3 cells at a concentration of 25 ng/ml. In contrast, A23187 at a concentration of 2.5 μM did not induce any detectable cytolysis against these tumours. However, in the presence of PMA (25 ng/ml) and A23187 (2.5 μM), a significantly higher level of lymphocyte-mediated cytotoxicity against these tumour targets was observed as compared to the cytolysis induced by PMA alone. Moreover, it was found that different tumour target cells showed different susceptibility to PMA and/or A23187 induced-cytolysis.

4.4 Determination of the cell type which is responsible for PMA and/or A23187 induced-lymphocyte-mediated cytotoxicity against tumours

In order to characterize the nature of effector cell population(s) which is responsible for mediating PMA and/or A23187-induced splenocyte cytotoxicity against tumours, T cells were depleted from normal mouse splenocytes by treatment with

Table 4.1 PMA and/or A23187- induced lymphocyte-mediated cytotoxicity against various tumours

Tumour target*	E:T@	% Specific ^{51}Cr release			
		Control	PMA	A23187	PMA+A23187
P815	50:1	0.9±0.7	5.1±0.9	0.5±1.1	7.8±1.0
	100:1	2.4±0.5	8.1±1.4	2.4±1.4	9.9±1.4
EL-4	50:1	2.0±0.2	4.2±0.3	2.1±1.5	6.6±1.1
	100:1	0.9±0.1	9.8±0.7	2.0±0.9	12.2±1.5
WEHI-3	50:1	0.9±0.2	5.8±1.3	2.1±0.8	16.4±1.2
	100:1	2.1±0.7	14.8±1.3	2.2±1.4	24.4±1.5

* Normal mouse splenocytes were preincubated with PMA (25 ng/ml) and/or A23187 (2.5 μM) at 37°C for 1 hr. Then ^{51}Cr-labelled tumour target cells were added and cytolysis was determined in a 10 hr ^{51}Cr release assay.

@ Effector-to-target cell ratio.

monoclonal anti-Thy 1.2 antibody and complement as described in Materials and Methods. The untreated or complement-treated groups served as the positive controls. Then these three groups of effector cells were preincubated with PMA and/or A23187 for 1 hr at 37 °C and then tested for splenocyte cytotoxicity against YAC-1 and MBL-2 cells. Data from Table 4.2 showed that 1. complement alone did not affect the PMA and/or A23187-induced lymphocyte-mediated cytotoxicity on both tumour targets; 2. the PMA- induced splenocyte cytotoxicity against both tumour targets diminished significantly by treatment with anti-Thy 1.2 antibody and complement; 3. the PMA+A23187-induced splenocyte cytotoxicity against YAC-1 cells was completely abrogated whereas the PMA+A23187 -induced cytolysis of the MBL-2 cells was significantly decreased by anti-Thy 1.2 antibody and complement treatment. These results, when taken together, indicate that the PMA and/or A23187- induced cytolysis is largely T-cell mediated.

4.5 Effect of T-cell enrichment by nylon wool column on PMA and/or A23187-induced lymphocyte-mediated cytotoxicity

To further establish the nature of effector cells involved in PMA and/or A23187-induced lymphocyte-mediated cytotoxicity, normal mouse splenocytes can be enriched for T cells by fractionation on nylon wool column as described in Materials and Methods. Both the unfractionated and the fractionated cells were preincubated with PMA and/or A23187 for 1 hr at 37°C. The ^{51}Cr-

Table 4.2 Determination of the cell type which is responsible for PMA and/or A23187-induced lymphocyte-mediated cytotoxicity**

Tumour target*	Group	% Specific ^{51}Cr release			
		Control	PMA	A23187	PMA+A23187
YAC-1	UNT	31.1±1.1	49.4±2.3	40.4±0.8	67.7±0.4
	C'	26.1±1.3	45.3±1.1	37.3±0.7	62.1±1.5
	Ab+C'	21.6±1.3	30.8±2.3	17.2±1.0	22.8±1.5
MBL-2	UNT	2.9±0.5	5.6±0.7	0.6±0.5	15.4±0.6
	C'	2.0±1.2	5.6±0.2	0	15.4±1.2
	Ab+C'	0.4±1.2	2.0±0.3	0	9.4±1.2

** Normal splenocytes were either untreated (UNT) or treated with complement alone(C') or with anti-Thy 1.2 antibody and complement (Ab+C') as described in Materials and Methods.

* For YAC-1 target, these three groups of splenocytes were preincubated with 6.3 ng/ml PMA and/or 0.63 μM A23187 at 37°C for 1 hr and then incubated with ^{51}Cr-labelled YAC-1 target cells for 6 hrs. For MBL-2 target, these three groups of splenocytes were preincubated with 25 ng/ml PMA and/or 2.5 μM A23187 at 37°C for 1 hr and then incubated with ^{51}Cr-labelled MBL-2 target cells for 10 hrs. The effector-to-target cell ratio was 100:1.

labelled YAC-1 and MBL-2 target cells were then added and the mixtures were further incubated for 6 hrs and 10 hrs respectively. It can be seen that the T cell-enriched, nylon wool nonadherent cells could be induced by PMA and/or A23187 to mediate significantly higher cytotoxicity against both tumour targets as compared to that of the unfractionated spleen cells (Table 4.3). In contrast, the nylon wool adherent cells, which contained mainly B cells and macrophages, were found to be much less effective in mediating cytolysis against the two tumour targets when stimulated by PMA and/or A23187. These results strongly indicate that the main effector cells responsible for the PMA and/or A23187-induced cytolysis are T cells.

4.6 Effect of pretreatment of splenocytes with PMA and/or A23187 on the induction of lymphocyte-mediated cytotoxicity against tumours

Previous experiments have shown that PMA and /or A23187 can induce normal mouse splenocyte cytotoxicity to lyse a variety of tumour targets. However, it should be noted that the two drugs were present continuously throughout the assay. It is of interest to determine whether a short preincubation of splenocytes with the two drugs followed by extensive washings can similarly trigger cytolysis. Therefore, normal splenocytes were preincubated with PMA and/or A23187 for 1 hr and then washed with plain RPMI medium for 3 times to minimize residual extracellular drugs. These PMA and/or A23187 pretreated splenocytes were then incubated with ^{51}Cr-labelled YAC-1 cells

Table 4.3 Effect of T cell enrichment by nylon wool column on PMA and/or A23187 induced lymphocyte-mediated cytotoxicity**

Tumour target*	Group	% Specific ^{51}Cr release			
		control	PMA	A23187	PMA+A23187
YAC-1	UNF	18.7+1.0	35.6+0.8	18.4+0.9	42.8+1.5
	NAF	23.1+0.2	60.6+0.9	32.3+1.7	78.7+2.6
	ADF	4.9+0.2	12.9+1.2	6.1+0.6	13.3+0.6
MBL-2	UNF	4.0+0.5	3.7+0.3	0.3+0.7	13.7+1.0
	NAF	3.5+0.4	5.3+0.5	1.0+0.5	17.1+0.1
	ADF	4.0+0.0	2.8+0.5	0.4+1.0	8.0+0.5

** Normal splenocytes were fractionated on nylon wool column into two fractions : the nonadherent fraction (NAF) and adherent fraction (ADF). The unfractioned cells (UNF) were included as a control.

* For YAC-1 target, these three groups of cells were preincubated with 6.3 ng/ml PMA and/or 0.63 μM A23187 at 37 °C for 1 hr and then incubated with ^{51}Cr-labelled YAC-1 cells for 6 hrs. For MBL-2 target, these three groups of cells were preincubated with 25 ng/ml PMA and/or 2.5 μM A23187 at 37 °C for 1 hr and then incubated with ^{51}Cr-labelled MBL-2 cells for 10 hrs. The effector-to-target cell ratio was 50:1.

and MBL-2 cells for 6 hrs and 10 hrs respectively. Data from Table 4.4 showed that a short pulse of normal splenocytes with PMA and/or A23187 was sufficient to trigger significant splenocyte cytotoxicity against the two tumour targets and the levels of cytolysis were similar to those induced by the continuous presence of PMA and/or A23187 throughout the assay.

4.7 The effect of temperature on the induction of lymphocyte-mediated cytotoxicity against tumours by PMA and/or A23187

In order to study the temperature requirement for induction of efficient lysis of the targets by PMA and/or A23187, normal splenocytes were preincubated with PMA and/or A23187 for 1 hr at 37 °C. The ^{51}Cr-labelled YAC-1 cells were then added and the mixtures were further incubated at 37°C, room temperature (22 °C) or 4°C for 6 hrs. Results in Table 4.5 showed that by cooling the cell mixture to 4°C or by maintaining the effector and target cells at room temperature through the whole incubation period, the induction of lymphocyte-mediated cytotoxicity against the YAC-1 tumour target by PMA and/or A23187 was completely inhibited.

Table 4.4 Effect of pretreatment of splenocytes with PMA and/or A23187 on the induction of lymphocyte-mediated cytotoxicity against tumours

Tumour target	E:T@	% Specific ^{51}Cr release			
		control	PMA	A23187	PMA+A23187
YAC-1*	50:1	12.7±2.1	18.8±0.9	10.2±0.7	28.9±2.0
	100:1	24.3±2.1	32.7±1.4	22.6±0.3	41.0±1.4
MBL-2**	50:1	3.2±0.8	8.9±1.5	4.0±0.3	14.0±1.4
	100:1	1.3±0.9	11.3±0.9	1.6±1.5	17.4±0.9

* Normal splenocytes were pretreated with 6.3 ng/ml PMA and/or 0.63 μM A23187 at 37 °C for 1 hr and then washed 3 times with plain RPMI medium. The PMA and/or A23187 pretreated splenocytes were then incubated with ^{51}Cr-labelled YAC-1 tumour cells for 6 hrs.

** Normal splenocytes were pretreated with 25 ng/ml PMA and/or 2.5 μM A23187 at 37 °C for 1 hr and then washed 3 times with plain RPMI medium. The PMA and/or A23187 pretreated splenocytes were then incubated with ^{51}Cr-labelled MBL-2 tumour cells for 10 hrs.

@ Effector-to-target cell ratio.

Table 4.5 The effect of temperature on the induction of lymphocyte-mediated cytotoxicity against tumours by PMA and/or A23187*

Tumour target	Incubation temperature	E:T@	% Specific ^{51}Cr release		
			Control	PMA	PMA+A23187
YAC-1	37°C	50:1	15.1±1.2	28.6±0.4	41.9±1.1
		100:1	23.7±0.5	37.9±0.4	54.7±0.9
	22°C	50:1	1.1±0.6	5.7±0.6	1.4±0.8
		100:1	2.2±0.5	9.7±0.2	3.4±0.7
	4°C	50:1	1.3±0.5	0	0.2±1.3
		100:1	0.9±0.9	0.8±1.0	0.3±0.7

* Normal splenocytes were preincubated with 0.63 ng/ml PMA and/or 0.63 μM A23187 at 37°C for 1 hr. The ^{51}Cr-labelled YAC-1 target cells were then added and the mixtures were incubated at the indicated temperature in an air-tight box containing 5% CO_2 in air. Cytolysis was then determined in a 6 hr ^{51}Cr release assay.

@ Effector-to-target cell ratio.

DISCUSSION

Recent studies have shown that phorbol ester, a potent activator of protein kinase C, when present alone or in combination with calcium ionophore, can mimic antigenic stimulation and is effective in triggering T cell activation (Isakov et al., 1986; Weiss and Imboden, 1987). In this chapter, the effect of PMA (a phorbol ester) and A23187 (a calcium ionophore) on triggering of normal mouse splenocytes to mediate cytotoxicity against various tumour targets was examined. It was found that PMA alone was able to trigger normal splenocytes to display a significant, albeit low, level of cytotoxicity against tumours. This nonspecific cytotoxicity was dependent on the concentration of PMA and could be observed with various tumour targets. Interestingly enough, others have found that treatment of murine or human cytotoxic T effector cells with phorbol ester alone can lead to either inhibition or enhancement or lytic activity, depending on which effector-target system is used, the duration of phorbol ester action and the concentration of drug that is used (Orosz et al., 1983; Bensussan et al., 1985; Russell, 1986; Schrezenmeier et al., 1986). On the other hand, it was found that calcium ionophore A23187 alone could not induce any detectable cytotoxicity against the tumour targets that had been tested. In contrast, it rather inhibited the endogenous NK activity of the splenocytes against YAC-1 cells at high concentrations. This finding is in line with a recent report (Mikael et al., 1987) which showed that a short term pretreatment of human lymphocytes with A23187 alone suppressed normal human NK

activity. The combination of the PMA and A23187, however, was found to induce much higher splenocyte killing against a variety of tumour targets, including the syngeneic (MBL-2, EL-4) and allogeneic (YAC-1, WEHI-3 and P815) tumour cell lines. Moreover, it should be noted that optimal induction of normal splenocyte cytotoxicity against two different tumour targets (i.e. MBL-2 and YAC-1 cells) required different combinations of the two drugs. The reason for such an observation is, however, unclear. Nevertheless, these results are quite similar to a number of recent reports which showed that these two drugs act synergistically to induce normal human lymphocytes and cloned murine cells to lyse a variety of tumour targets (Ju et al., 1987; Lancki et al., 1987; Mikael et al., 1987). Moreover, the present observations are consistent with other reports which showed that phorbol ester and calcium ionophore can cooperate synergistically to initiate different parameters of T cell activation such as expression of proliferation-related genes, cellular proliferation, lymphokine secretion and surface receptor expression (Albert et al., 1985; Trunch et al., 1985; Ju et al., 1987; Kumagai et al., 1987). Since PMA is an activator of PKC whereas A23187 can elevate intracellular Ca^{++}, such a synergism between PMA and A23187 strongly suggests that cooperation between PKC and intracellular Ca^{++} may be important in signal transduction during T cell activation and function.

Time course experiments showed that the PMA+A23187-induced splenocyte cytotoxicity against tumour targets was detectable at about 4 hrs of incubation and this increased progressively with

time. With YAC-1 target, it peaked at 10 hrs after incubation whereas with MBL-2 target, maximum cytolysis had not yet been reached even up to 12 hrs of incubation. The work with murine T cell clones demonstrated that the triggering of lysis by PMA+A23187 occurred much more rapidly, and a very short incubation period (about 3 hrs) was sufficient for the expression of maximal cytolytic activity (Ju et al., 1987; Lancki et al., 1987). These rapid kinetics indicate that the lytic activity induced by PMA and calcium ionophore may result primarily from the triggering of conventional lytic mechanisms rather than through activation of lymphokine production which requires a much longer period of incubation. It is known that PMA and A23187 can be used to activate a variety of cell types including macrophages and B cells (Bertoglio, 1983; Kiyotaki and Bloom, 1984; Drysdale et al., 1987). Therefore, in order to determine the main effector cell type(s) which is involved in the induction of splenocyte cytotoxicity by PMA+A23187, normal splenocytes were depleted of T cells by treatment with monoclonal anti-Thy 1.2 antibody and complement before preincubation with PMA and/or A23187. It can be seen that the PMA+A23187 induced cytolysis of tumour targets was markedly diminished by anti-Thy 1.2 antibody and complement treatment. Moreover, by fractionation of normal splenocytes on nylon wool column, it was found that the nonadherent cells (consisted of mainly T cells) but not the adherent cells (consisted of mainly B cells and macrophages) treated with PMA and/or A23187 expressed significant cytolytic activity. These data, when taken together, strongly suggest

that the PMA+A23187-induced splenocyte cytotoxicity was T cell-mediated. Similarly, Ju et al.(1987) found that only the $L3T4^+$ inducer T cells but not macrophages or B cells could be activated by PMA plus A23187 to express cytolytic function.

It is unclear how PMA and/or A23187 can induce normal mouse splenocytes to kill the syngeneic and allogeneic tumour cells. Therefore, it is of great interest to examine whether a brief exposure of normal splenocytes to PMA and/or A23187 can induce nonspecific lymphocyte-mediated cytotoxicity against tumours. The present results clearly showed that a brief exposure (1 hr) of splenocytes to PMA and/or A23187 was sufficient to activate them to kill the tumour targets nonspecifically. Whether pretreatment of the target cells alone has any effect on their subsequent sensitivity to lysis has not yet been investigated. Nevertheless, other studies have suggested that the action of PMA and/or A23187 is on the effector T cells rather than on the target cells (Russell, 1986; Schrezenmeier et al., 1986).

In the present study, the temperature requirement in the cytolysis induced by PMA and/or A23187 was also examined. It was found that PMA and/or A23187-activated splenocytes were lytically active only at 37°C. Cooling the incubation mixture to 4°C or by maintaining the effector and target cells at room temperature failed to induce any significant lysis. It is conceivable that temperature may affect PMA and/or A23187 -induced splenocyte cytotoxicity similar to that of LDCC (see Chapter 3) and CTL in general (Berke, 1980).

In conclusion, the present study merely demonstrated that PMA and/or A23187 treatment activates a lytic function in normal mouse splenocytes and the cytolysis observed is T cell-dependent. However, the mechanism(s) by which PMA and/or A23187 can trigger the normal mouse splenocytes to lyse tumour targets nonspecifically has not yet been elucidated. It would be of great interest to know whether the PMA and/or A23187-induced lysis requires close proximity or physical contact between the effector and target. In addition, whether the drug-induced lytic mechanism is identical to the mechanism activated after the physiological receptor-triggering of CTL awaits further investigation.

CHAPTER FIVE : GENERATION OF CYTOTOXIC T CELLS AGAINST SYNGENEIC TUMOUR BY *IN VITRO* IMMUNIZATION

CONTENTS

INTRODUCTION

Although it has been well established that the host immune system has the ability to reject syngeneic tumours,yet tumour-bearing animals often die from tumour progression despite the existence of anti-tumour immunity (Hellstrom and Hellstrom, 1969). This may reflect the presence of tumour-induced immunosuppression or inefficient *in vivo* sensitization of the host effector cells (Martin *et al.*, 1973; Fujimoto *et al.* 1976; Levy *et al.*, 1976; Small and Trainin, 1976). In fact, a number of previous reports showed that syngeneic or allogeneic cytotoxic cells against tumour targets could be generated by the *in vitro* immunization technique (Berke *et al.*, 1971; Cerottini *et al.*, 1974; Plata *et al.*, 1975; Glaser and Herberman, 1976; Kedar *et al.*, 1977; Cheever *et al.*, 1978). Splenocytes from normal, tumour-immune and tumour-bearing mice can also be used to generate cytotoxic cells with higher lytic ability. Anti-tumour activity of lymphoid cells can be generated *in vitro* that may be due to by-pass mechanisms that limit effective sensitization *in vivo* and tumour-induced immunosuppression (Martin *et al.*,1973; Treves *et al.*, 1975). Moreover, the *in vivo* anti-tumour activity of these cells *in vitro* generated have also been reported (Bernstein, 1977; Cheever *et al.*, 1978; Frenandez-Cruz *et al.*, 1979).

In our laboratory it was demonstrated previously that cytotoxic activity against the syngeneic MBL-2 tumour could be generated by *in vitro* stimulation of normal C57 BL/6J mouse

splenocytes with mitomycin C (MMC)-inactivated MBL-2 cells. (Wong, 1987). However, the inductive requirements, the antigen specificity and the nature of these cytotoxic effector cells have not been elucidated. Therefore, in this chapter, the effect of *in vivo* and *in vitro* immunizations on the generation of cytotoxic cells to a syngeneic tumour (MBL-2) was investigated. Moreover, the antigen specificity and the surface phenotype of these cytotoxic effector cells were also determined.

RESULTS

5.1 Effect of in vivo immunization with inactivated tumour on the in vitro generation of cytotoxic effector cells to syngeneic tumour

Previous work in this laboratory have shown that cytotoxic cells against syngeneic MBL-2 tumour can be generated *in vitro* by co-culturing normal C57 BL/6J mouse splenocytes with MMC-treated MBL-2 cells for 6 days (Wong, 1987). It is of interest to determine whether *in vivo* immunization with syngeneic inactivated tumour can enhance the subsequent induction of cytotoxic cells *in vitro*.

C57BL/6J mice were injected i.v. once (primary immunization) or twice (secondary immunization) with 10^7 MMC-inactivated MBL-2 cells. As it was reported that pretreatment of mice with cyclophosphamide (CY) could increase the ability of tumours to stimulate specific effector cells involved in anti-tumour immunity (Glaser, 1979; Hengst *et al.*, 1981; Ophir *et al.*, 1984), therefore, another two groups of mice were included and they were pretreated with CY (100 mg/kg body weight given i.p.) two days before primary or secondary immunization. In the control groups mice were similarly injected with the same volume of PBS. The primary immunized or secondary immunized mice were sacrificed 6 days and 3 days respectively after the last injection of tumour and the cytotoxic activity in their spleen cells was examined. As shown in Table 5.1 and 5.2, *in vivo* immunization alone failed to

Table 5.1 Effect of primary in vivo immunization on the in vitro generation of cytotoxic cells against a syngeneic tumour

Group	% Specific ^{51}Cr release from MBL-2 target			
	Before in vitro culture		After 5-day culture	
E:T@	25:1	50:1	12.5:1	25:1
Expt. 1 :				
NSC	3.4+0.4	6.1+0.4	12.1+1.3	14.9+1.1
ISC	5.7+0.4	5.6+0.3	30.3+1.6	43.4+0.5
CISC	4.9+0.3	4.9+0.3	51.9+2.2	65.2+0.7
Expt. 2 :				
NSC	2.3+1.1	3.9+0.5	12.9+1.2	18.9+0.8
ISC	5.9+0.6	6.2+0.4	53.4+2.6	65.4+1.6
CISC	5.5+0.8	5.8+0.8	66.1+3.3	76.6+2.8

* C57BL/6J mice in groups of 4 were injected i.v. either with PBS (NSC) or with 10^7 mitomycin C(MMC)-inactivated MBL-2 cells (ISC). A third group of mice were cyclophosphamide pretreated (100 mg/kg given i.p.) 2 days before i.v. injection with 10^7 MMC-inactivated MBL-2 cells (CISC Group). The mice were sacrificed 6 days after tumour immunization and their spleen cells were tested for cytotoxicity on MBL-2 target in a 8 hr ^{51}Cr release assay. In addition, the splenocytes from these 3 groups of mice were co-cultured with MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were again tested for cytotoxicity in a 8 hr ^{51}Cr release assay using labelled MBL-2 cells as target.

@ Effector-to-target cell ratio.

Table 5.2 Effect of secondary in vivo immunization on the in vitro generation of cytotoxic cells against a syngeneic tumour

Group*	% Specific ^{51}Cr release from MBL-2 target			
	Before in vitro culture		After 5-day culture	
E:T@	25:1	50:1	12.5:1	25:1
Expt. 1 :				
NSC	6.0+0.9	7.0+0.8	2.9+0.2	3.7+1.1
ISC	4.5+0.6	6.0+0.3	36.5+2.2	45.8+0.3
CISC	2.8+0.4	4.6+0.4	44.2+1.2	59.3+1.3
Expt. 2 :				
NSC	2.7+0.4	2.6+1.3	4.9+1.2	6.7+0.4
ISC	1.8+0.7	3.9+0.6	33.0+0.4	45.2+0.9
CISC	5.2+0.9	8.3+0.9	38.0+1.5	53.2+0.7

* C57 BL/6J mice in groups of 4 were injected i.v. on day 0 and day 21 either with PBS (NSC Group) or with 10^7 mitomycin C (MMC)-inactivated MBL-2 cells (ISC Group). A third group of mice were cyclophosphamide pretreated (100 mg/kg given i.p.) on day -2 followed by i.v. injection with 10^7 MMC-inactivated MBL-2 cells on both day 0 and day 21 (CISC Group). The mice were sacrificed 3 days after the second tumour immunization and their spleen cells were tested for cytotoxicity on MBL-2 targets in a 8 hr ^{51}Cr-release assay. In addition, the splenocytes from these 3 groups of mice were co-cultured with MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were again tested for cytotoxicity in a 8 hr ^{51}Cr-release assay using labelled MBL-2 cells as target.

@ Effector-to-target cell ratio.

elicit significant cytotoxic activity against MBL-2 cells, i.e., specific cytotoxic activity was always less than 10% even at an effector:target cell ratio of up to 50:1. However, when the immune spleen cells were co-cultured with MMC-inactivated MBL-2 cells for 5 days, a strong cytotoxic response was observed. It was found that similar levels of cytotoxicity were generated when the spleen cells were derived from primary or secondary immunized mice (Table 5.1 and 5.2). In contrast, much lower levels of cytotoxicity were generated *in vitro* when the spleen cells were derived from the unimmunized mice. Moreover, CY pretreatment slightly enhanced the cytotoxic response generated *in vitro* and so unless otherwise stated, mice were usually CY-pretreated in subsequent experiments.

5.2 Cross-reactivity of the cytotoxic cells raised against MBL-2 cells in vitro

In order to determine whether *in vitro* induction of cytotoxic response to a syngeneic tumour is dependent on the presence of the same tumour in the culture system, the normal or immune spleen cells from CY-pretreated mice (responders) were incubated for 5 days in the absence or presence of MMC-treated MBL-2 cells (stimulators) at a responder:stimulator ratio of 20:1. The results in Table 5.3 and 5.4 clearly showed that 1. very little cytotoxic activity against the MBL-2 target was generated *in vitro* by culturing normal spleen cells alone whereas a slightly higher level of cytotoxicity was detected when the normal spleen cells were stimulated with MMC-treated MBL-2 cells

Table 5.3 Cross-reactivity of the cytotoxic cells generated in vitro from primary immunized spleen cells

In vitro cell culture*			% Specific ^{51}Cr release from		
Responder cells	Stimulator cells	E:T@	MBL-2	EL-4	P815
CSC	-	10:1	4.3±0.2	1.5±0.7	2.5±1.4
		20:1	5.9±0.7	1.2±0.7	4.1±0.8
CSC	+	10:1	7.2±0.9	1.9±0.4	2.5±1.4
		20:1	9.4±0.2	1.7±0.4	2.9±1.2
CISC	-	10:1	18.3±0.6	9.0±0.6	4.6±1.4
		20:1	27.0±1.3	11.6±1.5	10.2±1.4
CISC	+	10:1	35.7±0.9	19.7±1.2	15.7±0.6
		20:1	49.5±0.9	31.1±3.4	24.1±0.6

* C57BL/6J mice in groups of 4 were cyclophosphamide pretreated (100mg/kg given i.p.) on day -2 and then followed by i.v. injection with either PBS (CSC) or with 10^7 MMC-inactivated MBL-2 cells (CISC) on day 0. The mice were sacrificed on day 6 and their spleen cells were co-cultured in the absence or presence of MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were tested for cytotoxicity in a 8 hr ^{51}Cr release assay using labelled MBL-2, EL-4 and P815 cells as targets.

@ Effector-to-target cell ratio.

Table 5.4 Cross-reactivity of the cytotoxic cells generated in vitro from secondary immunized spleen cells

In vitro cell culture			% Specific ^{51}Cr release from		
Responder cells	Stimulator cells	E:T@	MBL-2	EL-4	P815
CSC	-	10:1	6.7±1.2	2.0±0.3	3.1±1.7
		20:1	12.8±0.1	0.9±0.4	3.7±0.6
CSC	+	10:1	10.8±0.8	1.6±0.6	4.3±0.2
		20:1	20.0±0.4	1.5±0.2	4.3±0.3
CISC	-	10:1	26.3±1.8	10.2±1.1	20.8±1.2
		20:1	36.1±0.5	12.9±0.5	29.7±1.4
CISC	+	10:1	42.4±1.0	10.2±1.0	28.2±1.1
		20:1	54.8±0.6	10.3±1.7	33.6±0.5

* C57 BL/6J mice in groups of 4 were cyclophosphamide pretreated (100 mg/kg given i.p.) on day -2 followed by i.v. injection with either PBS (CSC) or with 10^7 MMC-inactivated MBL-2 cells (CISC) on both day 0 and day 21. The mice were sacrificed 3 days after the second tumour immunization and their spleen cells were co-cultured in the absence or presence of MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were again tested for cytotoxicity in a 8 hr ^{51}Cr release assay using labelled MBL-2, EL-4 and P815 cells as targets.

@ Effector-to-target cell ratio.

in vitro ; and 2. the in vivo MBL-2 primed immune spleen cells cultured without MMC-treated MBL-2 cells could spontaneously generate a significant level of cytotoxic activity against the MBL-2 target. However, a much higher level of cytotoxicity was attained in the presence of MMC-treated MBL-2 cells in vitro.

The antigen specificity of these cytotoxic effector cells generated in vitro was also tested using other tumour targets such as EL-4 and P815 cells. As shown in Table 5.3 and 5.4, both the EL-4 and P815 tumours were significantly lysed by the cytotoxic cells raised against the MBL-2 tumour, indicating that such cytotoxic cells did exhibit cross-reactivity with other tumour targets. Nevertheless, these effector cells showed significantly higher levels of cytotoxicity towards the MBL-2 tumour that was used for in vivo and in vitro immunizations.

The cross-reactivity of the cytotoxic cells generated in vitro was further investigated by examining their proliferative response to stimulation with different tumour antigens in vitro. The data in Table 5.5 and 5.6 showed that the cytotoxic cells raised against the MBL-2 tumour could also proliferate in response to stimulation with other tumour antigens, though maximum proliferation was always achieved in the presence of the specific MBL-2 antigens. Moreover, the effect of exogenous IL-2 on the proliferative response of the cytotoxic cells was also studied. It was found that 1. in the presence of 10% IL-2, there was a drastic increase in the rate of cell proliferation; and 2. the proliferative response was similar irrespective of the

Table 5.5 Proliferative response of the in vitro generated cytotoxic cells to various tumours

Group	Responder cells*	^{3}H-TdR incorporation (CPM ± S.E.) Stimulator cells			
		Nil	MBL-2	EL-4	P815
No IL-2	-	/	149±9	124±5	190±28
	+	1223±224	2910±392	1535±252	2045±32
With 10% IL-2	-	/	57±3	59±1	58±6
	+	51497±4801	46259±2251	44544±1152	48298±2008

* C57 BL/6J mice in groups of 4 were cyclophosphamide pretreated (100 mg/kg given i.p.) 2 days before i.v. injection with 10^7 MMC-inactivated MBL-2 cells. The mice were sacrificed 6 days after tumour immunization and their spleen cells were co-cultured with MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable cells were further cultured for 2 days in the absence of any stimulator cells. These responder cells were then co-cultured with various MMC-treated tumour stimulator cells at a responder:stimulator ratio of 5:1 (5×10^4 responders/well) in a 96-well flat-bottomed microtiter plate in the absence or presence of IL-2. Two days later, each well was then pulsed with 0.5 uCi ^{3}H-TdR for 16 hrs and the incorporated radioactivity was determined.

Table 5.6 Proliferative response of the in vitro generated cytotoxic cells to various tumours

Group	Responder cells*	^{3}H-TdR incorporation (CPM ±S.E.) Stimulator cells: Nil	MBL-2	EL-4	P815
No IL-2	-	/	37±3	30±1	48±4
	+	134±14	842±81	257±78	581±90
With 10% IL-2	-	/	39±3	34±3	31±2
	+	38846±2023	42346±1621	41883±3322	41748±1184

* C57BL/6J mice in groups of 4 were cyclophosphamide pretreated (100 mg/kg give i.p.) on day -2 followed by i.v. injection with 10^7 MMC-inactivated MBL-2 cells on both day 0 and day 21. The mice were sacrificed 3 days after the second tumour immunization and their spleen cells were co-cultured with MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were further incubated for 2 days in the absence of any stimulator cells. These responder cells were then co-cultured with various MMC-treated tumour stimulator cells at a responder:stimulator ratio of 5:1 (5 x 10^4 responders/well) in a 96-well flat-bottomed microtiter plate in the absence of presence of IL-2. Two days later, each well was pulsed with 0.5 uCi ^{3}H-TdR for 16 hrs and the incorporated radioactivity was determined.

absence or presence of tumour antigens.

5.3 Determination of the phenotype of the cytotoxic cells generated in vitro

As shown previously, cytotoxic cells can be obtained by *in vitro* stimulation of the *in vivo* primed immune spleen cells. The phenotype of these cytotoxic effector cells was determined by treatment of the effector cells with monoclonal anti-Thy 1.2 antibody plus complement as described in Materials and Methods. The untreated or complement-treated cells served as the positive controls. These three groups of effector cells were incubated with ^{51}Cr-labelled tumour targets including MBL-2, P815 and EL-4 cells and cytotoxicity was determined in a 8 hr ^{51}Cr-release assay. As shown in Table 5.7 and 5.8, complement treatment alone did not affect the cytotoxic activity of the effector cells whereas depletion of T cells by treatment with anti-Thy 1.2 antibody and complement greatly diminished the cytotoxic activity of the effector cells against various tumour targets, indicating that the cytotoxic activity was T-cell mediated.

Table 5.7 Elimination of the cytotoxic activity generated in vitro by anti-Thy 1.2 antibody and complement treatment

Treatment of effector cells with*	E:T@	% Specific ^{51}Cr-release from		
		MBL-2	EL-4	P815
Nil	10:1	35.7±0.9	19.7±1.2	31.1±3.4
	20:1	49.5±0.9	31.1±3.4	24.1±0.6
C'	10:1	31.3±3.2	19.8±1.2	13.7±0.6
	20:1	46.1±2.7	26.1±2.1	22.1±0.6
Anti-Thy 1.2 + C'	10:1	1.6±0.7	0.7±0.6	0.2±1.3
	20:1	1.2±0.6	0.2±0.4	0

* C57BL/6J mice in groups of 4 were cyclophosphamide pretreated (100 mg/kg given i.p.) 2 days before i.v. injection with 10^7 MMC-inactivated MBL-2 cells. The mice were sacrificed 6 days after tumour immunization and their spleen cells were co-cultured with MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were depleted of T cells by anti-Thy 1.2 antibody and complement treatment as described in Materials and Methods. The treated cells were then tested for cytotoxic activity against MBL-2, EL-4 and P815 target cells in a 8 hr ^{51}Cr release assay.

@ Effector-to-target cell ratio.

Table 5.8 Elimination of the cytotoxic activity generated in vitro by anti-Thy 1.2 antibody and complement treatment

Treatment of effector cells with*	E:T@	% Specific ^{51}Cr release from		
		MBL-2	EL-4	P815
Nil	10:1	42.5±1.0	10.2±1.0	28.2±1.1
	20:1	54.8±0.6	10.3±1.7	33.6±0.5
C'	10:1	40.4±0.8	9.5±1.7	31.3±2.2
	20:1	59.8±1.6	12.9±1.0	33.6±1.3
Anti-Thy 1.2 + C'	10:1	11.0±1.8	0.9±0.7	3.7±1.6
	20:1	12.9±0.6	0	2.5±1.2

* C57BL/6J mice in groups of 4 were cyclophosphamide pretreated (100 mg/kg given i.p.) on day -2 followed by i.v. injection with 10^7 MMC-inactivated MBL-2 cells on both day 0 and day 21. The mice were sacrificed 3 days after the second tumour immunization and their spleen cells were co-cultured with MMC-inactivated MBL-2 cells at a responder-to-stimulator cell ratio of 20:1 in 75 cm^2 tissue culture flasks for 5 days. After the removal of dead cells and debris by centrifugation on Ficoll-Isopaque gradient, the viable effector cells were depleted of T cells by anti-Thy 1.2 and complement treatment as described in Materials and Methods. The treated cells were tested for cytotoxic activity against MBL-2, EL-4 and P815 target cells in a 8 hr ^{51}Cr release assay.

@ Effector-to-target cell ratio.

DISCUSSION

Previous work in this laboratory have shown that cytotoxic cells against the syngeneic MBL-2 tumour can be induced *in vitro* by culturing normal C57BL/6J mouse splenocytes for 5-6 days in the presence of mitomycin C-inactivated MBL-2 cells (Wong, 1987). In order to establish the optimal conditions for the induction of MBL-2-reactive cytotoxic cells, the effect of specific *in vivo* and *in vitro* immunizations on the generation of cytotoxic activity against syngeneic MBL-2 tumour was examined. The present data showed that both primary and secondary *in vivo* immunized spleen cells did not exhibit any significant cytotoxicity against MBL-2 cells. However, when these immune spleen cells were co-cultured with MMC-inactivated MBL-2 cells for 5 days, high levels of cytotoxic activity against the syngeneic MBL-2 target were detected. In addition, the cytotoxicity generated from primary and secondary *in vivo* immunized splenocytes were of similar magnitudes. The present results are in line with others who found that the generation of in vitro cytotoxicity against tumours required both culture with specific tumours as well as previous *in vivo* priming (Bernstein *et al.*, 1976; Cheever *et al.*, 1978; Nakano and Masuda, 1983a,b). The failure to elicit a detectable cytotoxic response by *in vivo* immunization with MBL-2 cells may be attributed to the inadequate number of effector cells resulting from poor sensitization *in vivo*. However, this is considered to be less likely because 1) significant cytotoxic activity can be generated *in vitro* simply by culturing the primary immune spleen cells in the absence or presence of specific antigen; and 2) repeated *in vivo*

immunizations with a high dose of tumour still failed to induce a detectable cytotoxic response. Alternatively, the inability to generate a cytotoxic response to MBL-2 cells *in vivo* may be due to factors in the host which limit or interfere with the generation and/or expression of cytotoxic effectors, such as tumour-induced immunosuppression, formation of blocking antibodies and suppressor cells etc. Such a possibility is supported by the earlier finding that the MBL-2 tumour could induce both suppressor cells and suppressor factors *in vivo* (Wong, 1987). Thus it is conceivable that culturing of cells primed *in vivo* may by-pass suppressor mechanisms and resulted in the generation of potent cytotoxic activity *in vitro*. Moreover, it should be noted that a significant level of cytotoxicity against the MBL-2 target could also be observed by culturing the primary or secondary immune cells in the absence of exogenous tumour antigens, indicating the the presence of specific tumour *in vitro* may not be an absolute requirement for the induction of cytotoxic effector cells. However its presence may optimize the response by inducing specific clonal expansion of effector cells *in vitro*. In addition, it was found that pretreatment of mice with cyclophosphamide before *in vivo* immunizations slightly enhanced the cytotoxic response generated *in vitro*. How CY can enhance the effect of *in vivo* priming is as yet unclear. It was suggested that administration of CY prior to immunization might result in nonspecific elimination of suppressor T cells and thus stronger anti-tumour response could be developed (Glaser, 1979; Hengst *et al*., 1981).

In order to determine the specificity of the cytotoxic response generated *in vitro*, different tumour cells including P815 and EL-4 cells were used as targets. It can be seen that the cytotoxic cells derived from *in vitro* restimulation of *in vivo* primed cells could efficiently lyse these 'third-party' tumour targets although these activated cells showed much higher cytotoxicity against the MBL-2 cells that were used for *in vivo* and *in vitro* immunizations. A cross-reactivity in the *in vitro* generated cytotoxic cells was also reported by other investigators (Nakano and Masuda, 1983a,b). Moreover, the cross-reactivity of the cytotoxic cells generated *in vitro* was also detected in their proliferative response to stimulation with different tumour antigens *in vitro*. In the absence of exogenous IL-2, the cytotoxic cells demonstrated maximum proliferative response to stimulation with MBL-2 tumour antigens, yet they also proliferated in response to other tumour antigens. However, in the presence of 10% IL-2, the cytotoxic cells become greatly dependent on IL-2 for proliferation and their specific response to tumour antigens could no longer be demonstrated. These results, when taken together, indicated that the cytotoxic effector cells generated *in vitro* were quite heterogeneous in nature and they exhibited some nonspecific cytotoxic activity against other tumours. Moreover, the ability of these cytotoxic cells to proliferate rapidly in response to IL-2 strongly suggested that *in vitro* immunization might also induce the expression of IL-2 receptors on these cultured cells.

As an initial attempt to characterize the nature of these cytotoxic cells, they were depleted of T cells by treatment with monoclonal anti-Thy 1.2 antibody and complement. It was found that the cytotoxic activity against various tumour targets was markedly diminished by depletion of T cells, indicating that the cytotoxic activity generated *in vitro* was largely T-cell mediated. The high reactivity of the cytotoxic cells towards the MBL-2 tumour strongly suggested that the effector cell might contain MBL-2-specific cytotoxic T lymphocytes. However, since cross-reactivity was unambiguously demonstrated with other tumour targets, it is conceivable that the effector cells might also contain other types of nonspecific killer cells. It has been known that cytotoxic cells which nonspecifically lyse a variety of targets including the autologous tumour cells can also be generated *in vitro* by culturing lymphocytes with autologous tumours or in the presence of IL-2. These cells have been referred to as 'anomalous killers', 'natural-killer-like cells', 'activated lymphocyte killer (ALK) cells' and 'lymphokine activated killer (LAK) cells' (Herberman *et al.*, 1987; Hersey and Bolhuis, 1987). At present, how many types of cytotoxic cells are activated by *in vitro* immunization with MBL-2 tumour have yet to be resolved. The results so far suggested that NK or NK-like cells are unlikely to be the major cell type responsible for the *in vitro* cytotoxicity as two NK-insensitive tumour targets (i.e. P815 and EL-4 cells) were killed efficiently by cytotoxic cells generated *in vitro*. Moreover, the *in vitro* generated cells were found to be highly sensitive to anti-Thy 1.2 plus complement treatment whereas NK cells were reported to be much less

sensitive to such a treatment (Jondal, 1987). On the other hand, the cytotoxic cells were generated *in vitro* in the absence of any exogenous IL-2, suggesting that the LAK cells might not be involved in the observed cytotoxicity against tumours. Nevertheless, it is still possible that the endogenous IL-2 produced by stimulation with MBL-2 antigens might activate the cultured cells to become LAK cells. Furthermore, the cytotoxic cells generated *in vitro* might also contain the so called ALK cells as this type of cytotoxic cells are MHC-unrestricted in their lytic activity and they all carry the T cell marker (Hersey and Bolhuis, 1987).

In order to dissect the heterogeneity of these cytotoxic T cells, cloning methods have to be introduced so as to identify the cytotoxic subset(s) of lymphocytes and to determine the nature of tumour antigens recognized by the activated lymphocytes. More recently, a few long-term IL-2 dependent T cell lines have been established by repetitive *in vitro* restimulation of the cytotoxic cells with MBL-2 antigens in the presence of 10% IL-2. Although these cells lines have retained antigen-specificity in their proliferative response to stimulation with MBL-2 tumour antigens, yet their cytotoxic activity seemed to decrease gradually upon long term culturing (data not shown). It has been suggested that the lytic activity of some cytotoxic T cells may be rapidly downregulated by long term culture in the presence of IL-2 (Shih and Truitt, 1987). Whether a similar feedback mechanism is operative in our system remains to be established.

GENERAL DISCUSSION

The most important tool employed in my research project for measuring cytotoxicity against tumours is the ^{51}Cr-release assay which determines the percentage lysis of tumour cells. This technique was first introduced by Brunner et al. (1968) for the cell-mediated lytic assays. The mechanism of the ^{51}Cr release assay can be illustrated as follows. ^{51}Cr enters into the target cells and binds to the negatively charged cytoplasmic proteins tightly during the labelling of target cells with ^{51}Cr. Therefore, only when the cell membranes of ^{51}Cr-labelled target cells are damaged seriously will the ^{51}Cr-labelled proteins (80kd-125kd) be released into the extracellular medium and their associated radioactivity can then be determined. It has been reported that the percentage of ^{51}Cr release is proportional to the percentage of cell lysis (Berke, 1980; Schlager and Adams, 1983). The ^{51}Cr releasse assay has the advantages of the ability to handle large number of samples and to scale down the cytotoxicity test so as to reduce the quantities of reagents used as well as having greater precision. However, it exhibits early and high spontaneous release with most tumour targets so it is unsuitable for long incubation assays. Moreover, it cannot be used at low target cell concentrations. There are other techniques for monitoring the viability and disintegration of cells (Vaage and Agarwal, 1978; Schlager and Adams, 1983). One of these techniques is the trypan blue dye exclusion method. The basis of this method is that only dead cells will take up the dye whereas viable cells will exclude the dye. Therefore, dead cells

and viable cells can be distinguished clearly when viewed through the microscope. Although this method is simple, rapid and involves minimal manipulation of cells, however, it has an inherent standard error due to the practical limit to the number of cells that can be counted under the microscope. Moreover, it is unreliable for quantitation of target cell death in a cell-mediated cytotoxicity assay because the dead effector and target cells will both be stained and they can hardly be distinguished under the microscope. On the other hand, the ^{3}H-thymidine release method has often been used in most long-term cell-mediated cytotoxicity assays. However, the time required for labelling target cells with ^{3}H-thymidine is quite long. Moreover, the release of ^{3}H-thymidine-labelled DNA requires the destruction of nuclear envelope. Therefore, it is insensitive for short term assays. Based on the above considerations and since most experiments in my thesis work require a sensitive technique for the determination of percentage lysis of tumour cells within 6-10 hrs, the ^{51}Cr release assay was deliberately chosen for my study.

In contrast to previous reports (Forman and Moller, 1973; Bevan and Cohn, 1975; Bonavida and Bradley, 1976; Bradley and Bonavida, 1978), it was found that normal mouse splenocytes could be induced to lyse both syngeneic and allogeneic tumour cells in vitro by lectins. The lectins tested include PHA, Con A and its succinylated derivative (sCon A). How these lectins can trigger nonspecific cytotoxicity in normal mouse lymphocytes is as yet

unclear. All these lectins are known to be mitogenic to T cells. Moreover, they can also cross-link effector and target cells. Our data so far indicate that the activation of normal splenocytes alone by mitogenic lectins may not be sufficient to induce cytolysis of tumours as it was found that pretreatment of effector cells with sCon A for 1 hr failed to induce significant lymphocyte-mediated cytotoxicity. However, whether cross-linking of effector-to-target alone is sufficient for cytolysis to occur has not yet been determined. For future investigations, non-mitogenic plant lectins such as wheat germ agglutinin (WGA), peanut agglutinin (PNA) and soyabean agglutinin (SBA) or agents such as poly L-lysine, which readily agglutinate effector and target cells, should be examined for their ability to induce normal splenocytes to kill tumour targets *in vitro*.

Previous work had shown that increasing concentrations of succinyl Con A, unlike that of Con A, did not inhibit membrane mobility or lymphocyte proliferation (Hadden *et al.*, 1976). Similarly, the present investigation showed that Con A but not sCon A exhibited diminished ability on induction of splenocyte cytotoxicity against YAC-1 and MBL-2 targets at higher concentrations. Why Con A and sCon A exhibited different dose-response curves on induction of nonspecific lymphocyte-mediated cytotoxicity is not entirely clear. However, it was reported that Con A could raise intracellular cAMP level at high concentrations whereas the same concentration of sCon A had no appreciable effect on cAMP level (Hadden *et al.*, 1976). It has well been established that cAMP can suppress the lytic ability of

specific CTL as well as its proliferation. cAMP may also play a role in regulating the lytic apparatus of lymphocytes. Therefore, it would be of great interest to examine whether cAMP can suppress the lectin-induced cytolysis of tumours. This can be achieved by using drugs such as cholera toxin that can raise intracellular cAMP level and its effect on the induction of LDCC will then be evaluated. Moreover, whether cGMP may also play a role in regulating lectin-induced cytolysis is worth investigating.

Our data have shown that different tumour targets have different susceptibility to lectin-induced cytolysis. These results seem to indicate that lectin interaction with target cell structure is required for LDCC to occur. However, it is known that lectins can bind to the carbohydrate portion of several distinct cell surface glycoproteins on either the effector or target cells . Therefore, it is still uncertain whether the lectins work 'exclusively' by modifying surface structures on the effector cell, the target cell or on both cells. A recent report has shown that Con A must act on a papain-sensitive, N-glycosylated surface component to render target cell susceptible to CTL-mediated lysis (Gorman et al., 1987). Whether there is a similar target cell structure requirement in the lectin-induced cytolysis in our system is unknown. Moreover, the importance of MHC antigens and other surface molecules on the effector and target cells in mediating LDCC has yet to be defined.

Although the present work clearly demonstrated that lectins can induce normal splenocytes to lyse both syngeneic and allogeneic tumours. However, the *in vivo* effect of lectins on the growth of tumours has not yet been investigated. Kataoka *et al.* (1983) had shown that Con A-treated tumour cells could induce stronger tumour-specific immunity than untreated tumour cells when injected into syngeneic animals. Interestingly enough, it has been reported that tumour cells preincubated with animal lectins *in vitro* had diminished ability to grow *in vivo* (Yamazaki *et al.*,1983). In view of these findings, it would be of great interest and importance to examine whether lectins can exert an anti-tumour effect *in vivo*. Therefore in our future experiments, normal splenocytes pretreated with lectins *in vitro* will be examined for their ability to prolong the life of tumour-bearing animals upon adoptive transfer. In addition, tumour cells pretreated with lectins *in vitro* will be tested for their susceptibility to lymphocyte-mediated killing *in vivo*. Moreover, the ability of lectin-treated tumours to induce a greater immune response in the host will also be evaluated.

In the present study, it was found that PMA but not A23187 alone was able to trigger normal splenocytes to mediate cytolysis against various tumour targets. In addition, PMA and A23187 synergistically activated normal splenocytes to mediate nonspecific cytotoxicity. This synergism has been suggested to be related to the Ca^{++} and PMA activation requirements of protein kinase C (PKC). Although there is evidence to suggest that most, if not all, effects of phorbol esters are mediated through

activation of PKC (Nishizuka, 1984; 1986), nevertheless, it has been reported that phorbol ester such as PMA has more effects than just PKC activation, including modification of T cell surface antigens (Bensussan et al., 1985) or acting as a membrane fusigen or perturber (Nishizuka, 1984). Therefore, in order to establish that PKC activation is essential in triggering normal splenocytes to lyse tumour targets, two different experimental approaches can be adopted. One approach is to measure the PKC activity directly in cells that are activated with PMA and/or A23187. Alternatively, the effect of other pharmacologic agents such as dihydroteleocidin B (a tumour promoter that can activate PKC but is structurally unrelated to PMA) and 4-alpha-phorbol-12,13-didecanote (a phorbol ester analogue which neither promotes tumours nor activates PKC) on the induction of splenocyte cytotoxicity against tumours should be examined.

The work in this thesis have shown that a short term incubation of normal spleen cells with PMA and/or A23187 can lead to significant lysis of various tumour targets, however, the effect of long term incubation with these drugs on the induction of cytolysis has not yet examined. Interestingly, others had demonstrated that short term incubation with murine and human CTL with phorbol ester would result in significant triggering of CTL whereas prolonged incubation had a negative effect on the CTL lytic capacity (Russell, 1986; Schrezenmeier et al., 1986). Whether a similar effect will be seen with the normal mouse

splenocytes has yet to be determined.

The present investigation showed that the cytotoxic activity was mediated by T-cell in both lectin- and drug- induced cytolysis. However, which T-cell subset plays a major role in these types of cytotoxicity remains obscure. A recent report showed that the LDCC activity exhibited by isolated OKT8 T cells was found to be supperior to that by $OKT4^{+}$ T cells and unfractionated T lymphocytes (Perl *et al.*, 1984). Therefore in future experiments, fractionation of the normal spleen cells into helper and suppressor populations or depletion of specific T cell subsets by monoclonal antibody and complement treatment may provide better insights into the nature of effector cells mediating nonspecific cytotoxicity against tumours. Moreover, other reports have shown that murine macrophages can be activated by phorbol ester and calcium ionophore to kill tumour cells (Somers *et al.*, 1986). Whether lectins may also induce macrophages to kill tumour cells *in vitro* remains to be seen.

The generation of cytotoxic T cells against a syngeneic tumour (MBL-2) by *in vitro* immunization of tumour-immune cells has been established in our laboratory. It was found that primary or secondary *in vivo* immunized cells rather than normal cells could be stimulated *in vitro* to develop potent cytotoxic activity against the syngeneic MBL-2 tumour. In addition, pretreatment of mice with cyclophosphamide before immunization could enhance the anti-tumour ability of effector cells generated *in vitro*. These cytotoxic cells were found to be T-cells.

However, they also exhibited cross-reactivity with other tumours. These results suggest that, these cytotoxic cells are likely to be heterogeneous in nature and they may contain other cell types such as LAK cells or ALK cells other than specific CTL. In order to dissect the heterogeneity of these cytotoxic cells, cloning of these effector cells is essential. Our preliminary work showed that these cytotoxic cells could be maintained in vitro for more than 3 months by repetitive in vitro restimulation with sonicated MBL-2 tumour antigens in the presence of 10% IL-2. Although these cells seemed to have retained the T cell marker and antigen specificity, their lytic activity decreased gradually upon long term culture. As it has been suggested that a downregulation in the lytic activity in CTL may be induced by long term exposure to IL-2 (Shin and Truitt, 1987), modifications in the culture procedures may be necessary. Suggestions include the alternate culturing of effector cells in the presence of either recombinant IL-2 or tumour antigens and the use of intact tumour cells for long term stimulation. Using such strategies it is hoped that tumour-specific T cell clones can be established in our laboratory in the forseeable future.

In conclusion, our results so far suggest that normal mouse T lymphocytes can be triggered by different agents (specific tumour antigens, lectins and pharmacologic agents) to exhibit specific or nonspecific cytotoxic activity against tumours in vitro. It would be of great interest to know whether the same signal transduction pathways are set in motion by the multiple

activating agents. Moreover, whether there is any parallel between LDCC and drug-induced killing and whether PMA and/or A23187 can synergize with plant lectins in inducing splenocyte cytotoxicity are intriguing aspects that remain to be explored. Finally, whether these cytotoxic cells generated *in vitro* can demonstrate similar anti-tumour activity *in vivo* awaits further investigation.

ABSTRACT

Mitogenic plant lectins such as PHA, Con A and succinyl Con A (sCon A) were found to induce significant splenocyte cytotoxicity against a variety of syngeneic and allogeneic tumour targets. The data showed that both Con A and sCon A were much potent than PHA for the induction of lectin-dependent cellular cytotoxicity (LDCC). sCon A differed from Con A in that its ability to induce LDCC was not diminished at higher lectin concentrations. Moreover, different tumour targets showed different susceptibility to lectin-induced cytolysis. Cytolysis was found to be T cell-mediated and dependent on the presence of Ca^{++} and appropriate temperature (37 °C). The question remains open as to what the lectin is providing in the cytotoxic assay. The results clearly showed that pretreatment of effector and/or target cells with Con A but not sCon A could induce significant LDCC against the MBL-2 and YAC-1 tumours. In addition, cytolysis was abolished by the addition of α-methyl-D-mannoside to the assay system indicating that the binding of lectins to the effector and/or target cell surface may be critical for lysis to occur. Furthermore, several pharmacologic agents such as H7 (an inhibitor of protein kinase C) and W7 (a calmodulin antagonist) were found to be inhibitory to LDCC suggesting that the lectin-induced cytolysis may also depend on signal transduction pathways to effect target cell lysis.

It has been known that phorbol ester, a potent activator of protein kinase C, when present alone or in combination with

calcium ionophore, can mimic antigenic stimulation and is effective in triggering T cell activation. The present work showed that PMA (a phorbol ester) but not A23187 (a calcium ionophore) alone was able to trigger normal mouse splenocytes to display a significant, albeit low, level of cytotoxicity against tumours. This nonspecific cytotoxicity was dependent on the concentration of PMA and could be observed with various tumour targets. In addition, the PMA- induced cytotoxicity was found to be significantly enhanced with the stimultaneous presence of A23187. Time course experiments showed that the PMA and/or A23187-induced splenocyte cytotoxicity occurred rapidly and significant cytolysis of tumour targets was detected at 4 hrs after incubation of effector and target cells. The rapid kinetics indicate that the cytolysis induced by PMA and/or A23187 may result primarily from the triggering of conventional lytic mechanisms rather than through activation of lymphokine production. A brief exposure of the effector cells to PMA and/or A23187 was found to be sufficient to induce cytolysis and the effector cells mediating cytotoxicity were characterized to be T lymphocytes.

The effect of *in vivo* and *in vitro* immunizations on the generation of specific cytotoxic cells to a syngeneic tumour (MBL-2) was investigated. It was found that potent cytotoxic T cell activity directed against the MBL-2 tumour could be induced by stimulation of the *in vivo* primed spleen cells for 5 days in the presence of mitomycin C-treated MBL-2 tumour *in vitro*. The data showed that the cytotoxic activity generated from the

primary or secondary *in vivo* immunized splenocytes were of similar magnitudes. In addition, pretreatment of mice with cyclophosphamide before *in vivo* immunization slightly enhanced the cytotoxic response generated *in vitro*. Moreover, a cross-reactivity in the cytotoxic cells generated *in vitro* was demonstrated by their ability to lyse other tumour targets as well as their capacity to proliferate in response to stimulation with other tumour antigens *in vitro*. Collectively, these results indicate that the cytotoxic cells generated *in vitro* may be quite heterogeneous in nature. Therefore, cloning of these cells is required before they can be studied for their anti-tumour activity *in vivo*.

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